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*Journal of the American Association of Clinical Chemists*

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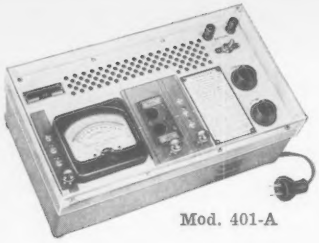

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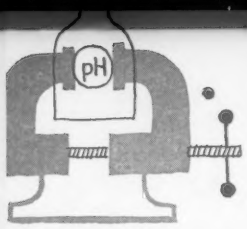


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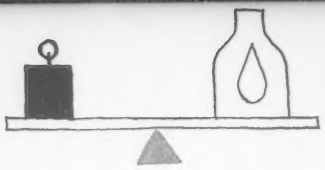

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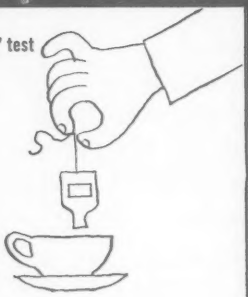

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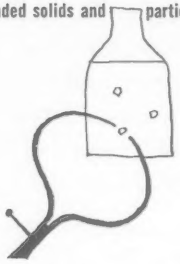

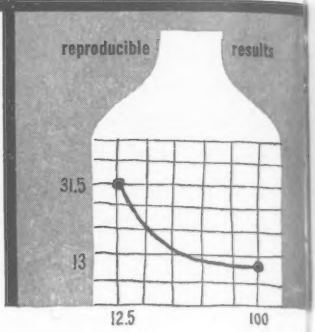


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# CLINICAL CHEMISTRY

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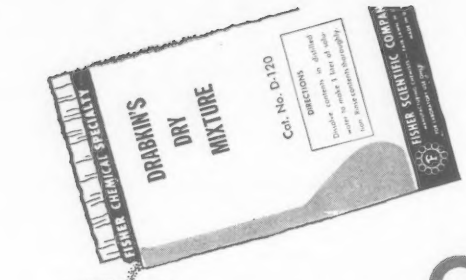


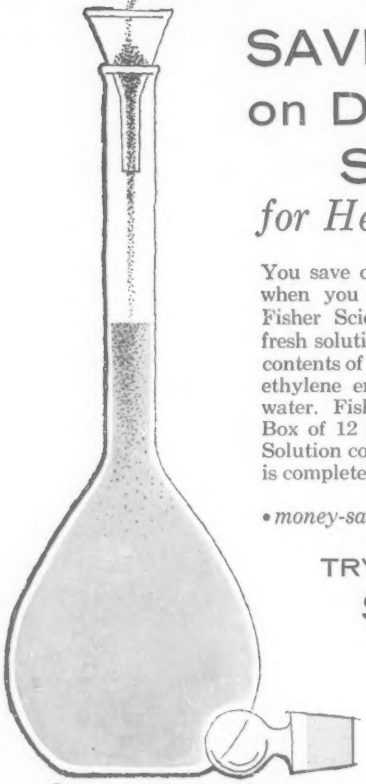

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# Direct Nesslerization of Blood and Urine Filtrates in the Determination of Urea (as Ammonia)

*Joseph H. Boutwell, Jr.*

THE SPECIAL REQUIREMENTS of a clinical laboratory are such that direct nesslerization for the determination of urea (as ammonia) in blood and urine filtrates is often preferred over procedures involving aeration or distillation to isolate the ammonia for subsequent analysis. It has long been recognized that direct nesslerization gives results with a lower order of accuracy and precision than those involving aeration or distillation. Numerous modifications have been resorted to by many investigators in an attempt to minimize turbidity effects in the nesslerized solutions. A review of some of these procedures is given by Gentzkow (1).

Some confusion has been evident in these attempts to eliminate turbidities, apparently because of a failure to recognize, as did Folin (2), the two sources of turbidity: increasing particle size of the Nessler's ammonia complex colloid, and a chemical reduction of the alkaline Nessler's reagent by nonammonia substances present in blood filtrates and in urine.

Recently, several methods have pointed toward the use of oxidizing agents to minimize the effect of these reducing agents on alkaline Nessler's reagent. Barrett (3), in 1935, advocated the use of sodium hypochlorite added to the alkaline Nessler's reagent just before use, and stated that persulfate and iodine gave similar results. Gentzkow, in 1942 (1), recommended the use of a combination of potassium persulfate and potassium gluconate. None of these modifications has achieved great popularity, probably because of the increased complications of unstable reagents, the failure to obey Beer's law and the easily observable difference in sub-

From Temple University School of Medicine and Temple University Hospital, Laboratory of Clinical Chemistry. Philadelphia, Pa.

Present Address: % Naval Medical School, National Naval Medical Center, Bethesda, Md.

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jective color between nesslerized standards and nesslerized blood filtrates. Gentzkow attempted to minimize the effect of this color difference by measuring optical densities at the isobestic point, which he determined to be between 490 and 510 mμ. All the aforementioned methods result in some decrease in the amount of color produced by a given amount of ammonia.

In 1954, we reported (4) that iodine would inhibit the turbidity produced by the interaction of alkaline Nessler's reagent and creatinine or uric acid. In 1955, Connerty *et al.* (5) recommended the use of an $I_2 \cdot KI$ solution. This present work is a continuation of our investigation of this iodine effect and a utilization of it in the photometric determination of blood and urine urea and urine ammonia.

In the development of these methods, certain collateral problems arose: the conditions affecting the colloidal stability of the Nessler's-ammonia complex; the effect of incomplete removal, by the usual deproteinization procedures, of certain substances precipitable by alkaline Nessler's reagent, which are thus capable of producing turbidity. This is an effect separate from that of the reducing substances (creatinine, uric acid, etc.) mentioned above. Urease preparation and stability in solution also proved to be a problem.

## PROBLEMS AND THEIR SOLUTIONS

In preliminary experiments it has been established that iodine added to alkaline Nessler's solution before use was not as effective in preventing the turbidity due to creatinine as elemental iodine added to the ammonia-containing solution before alkalinization. It had also been established that it would not be possible to add the desired amount of elemental iodine in the form of an aqueous solution in potassium iodide because of the effect of excess potassium iodide in decreasing the amount of color produced by a given amount of ammonia and by decreasing the range of linearity of the chromogenic reaction.

Finally, Johnson's device (9) of separating the Nessler's salt solution and the alkali and carrying out the nesslerization in two stages was resorted to. This procedure gives two advantages: (a) independent variation of the concentration of Nessler's salt and of the alkali, so as to achieve maximum stability with respect to both, is now possible, and (b) dissolving excess iodine in the Nessler's salt solution provides a simple answer to the problem of adding iodine to the nonalkalinized sample.

Preparation of the reagents is outlined below under the *urea method*. All optical measurements were made using matched test tube cuvets 15

mm. x 125 mm. through a Corning glass filter with maximum transmittance at 515 mμ in a modified Evelyn-type photometer.

### Optimal Concentration of Alkali and Nessler's Salt

Five milliliters of a solution containing 0.05 mg. of ammonia nitrogen were nesslerized by first adding Nessler's salt solution, mixing completely. After this, the alkali was rapidly added by syringe pipet, so as to give immediate, complete mixing. The optical densities were determined at 15 minutes and at 180 minutes. The ratio of the densities ($D_{180'}/D_{15'}$) for each tube was plotted as in Fig. 1. From these data the concentrations of alkali and Nessler's salt giving the highest degree of colloidal stability were selected for further work. The concentrations finally chosen were 1.0 ml. of Nessler's salt solution and 1.0 ml. of 2.5N NaOH (equivalent to the 1.25 ml. of 2.0N NaOH shown in Fig. 1) as being the most convenient and suitable for a 5-ml. sample. If the amount of iodine dissolved

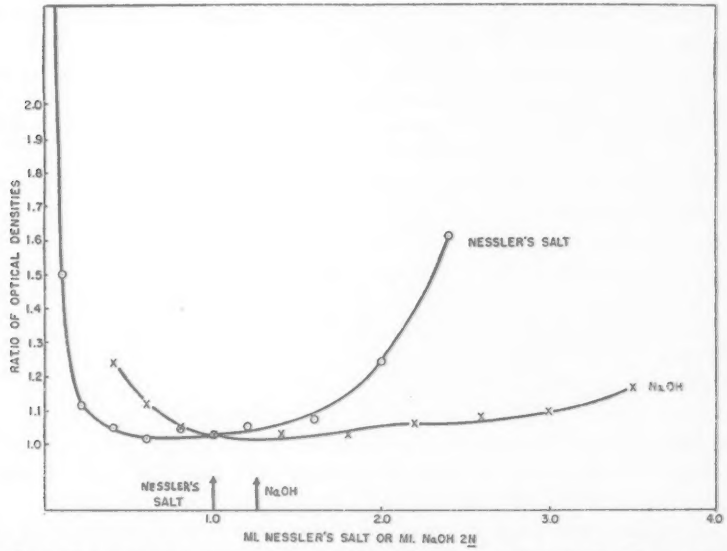


**Fig. 1.** Determination of the concentration of Nessler's salt and of alkali for maximum stability of the Nessler's-ammonia complex colloid.

Ordinate—the ratio of optical density at 180 minutes to that at 15 minutes ($D_{180'/15'}$).

Abscissa—the amount of reagent (Nessler's salt or alkali) added, in milliliters.

The *vertical arrows* indicate the concentrations finally chosen for further use (1.0 ml. of 2.5N NaOH was substituted for the equivalent 1.25 ml. of 2.0N NaOH shown in the figure).

in the Nessler's salt solution is changed, the optimal concentrations are altered.

### Comparison of the Protective Effect of Various Procedures Against Creatinine Turbidity

Creatinine was chosen as a convenient pure substance with which to measure the effectiveness of the various protective measures. The methods tested include: regular Nessler's salt; iodinated Nessler's salt; 2% iodine–potassium iodide solution—Connerty (5) procedure; Gentzkow (1) procedure; NaOCl—Barrett (3) procedure (using 0.25 mg. NaOCl); NaOCl—10x (using 2.5 mg. NaOCl). All these methods (with the exception of the Gentzkow procedure) were tested by the two-stage nesslerization technic described in the previous section.

After alkalinization, in the presence of creatinine, there is first a lag period following the addition of the alkali during which no turbidity is seen, and then a yellowish turbidity suddenly appears and rapidly increases. The time between the addition of the alkali and the appearance of the first visible turbidity was noted and plotted against the concentration of creatinine. The result is a hyperbolic curve (except in the case of Gentzkow procedure) with foci at varying distances from the origin. If 100/time (seconds) is plotted against the amount of creatinine, the abscissal intercept will represent the amount of creatinine given complete (or infinite time) protection (Fig. 2).

When the results of this procedure using the Gentzkow technic are plotted, the curve and the reciprocal plot are markedly different from those of other procedures used. This result is probably due to the fact that in this one procedure a premixed alkaline Nessler's reagent was used.

Table 1 lists the infinite time (complete protection) intercept for the various procedures in the absence and in the presence of 0.2 mg. ammonia nitrogen. These results clearly show the much greater protective effect of the two-stage iodinated Nessler's technic. Attempts to increase the effectiveness of $I_2 \cdot KI$ or of NaOCl by increasing the amount of the reagent used proved impractical because of decreased color formation and also (in the case of NaOCl) because of a reaction with Nessler's salt solution which resulted in the precipitation of mercuric iodide.

Creatinine was used in most of these experiments because of the difficulty in obtaining neutral or acid solutions of uric acid. In tests using uric acid, it was measured out as a freshly prepared alkaline solution. HCl 1.0N was then added to neutrality, and the suspension of precipitated uric acid was immediately nesslerized by the two-stage procedure.

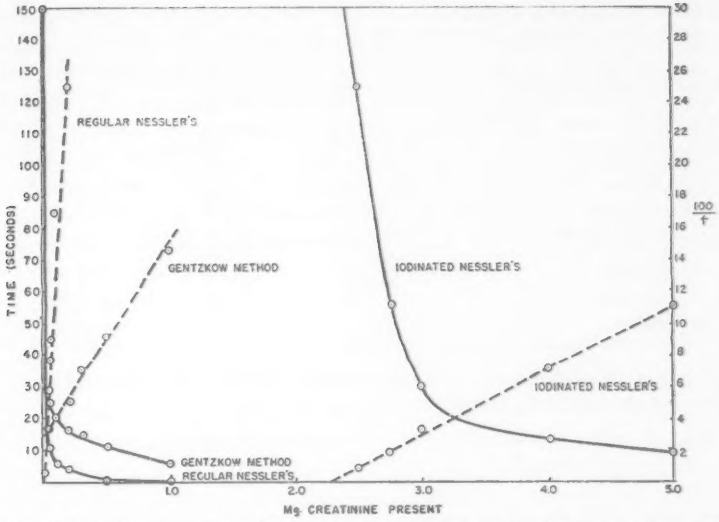


**Fig. 2.** Turbidity-time curves showing the relationship between the amount of creatinine present and the time required to develop turbidity. The solid lines show the plot of turbidity time versus concentration; the dashed lines show the plot of 100/time versus the concentration of creatinine.

### Linearity and Sensitivity

In Table 2 the relationship between optical density and the amount of amonia nitrogen nesslerized is shown. The constancy of the K values shows the excellent linearity even at optical densities of 1.5 or higher, corresponding to transmittances as low as 3 per cent. Regular Nessler's and iodinated Nessler's salt solutions (each used in the two-stage technic) show almost identical results. Addition of $I_2 \cdot KI$ (0.05 ml. of 2 % iodine as in the Connerty technic) depresses the sensitivity only slightly but 0.25 mg. of NaOCl (as in the Barrett procedure) decreases the sensitivity by about 20 per cent.

### Deproteinization Procedure

In the attempt to use the two-stage iodinated Nessler's technic in the determination of blood urea, it was noted that a slight turbidity slowly developed in some of the nesslerized solutions. This was most noticeable 2 to 3 hours after color development and was present only in nesslerized blood filtrates or in urea standards, not in nesslerized ammonia standards. It was felt that this turbidity represented some nitrogenous material

**Table 1.** Relative Effectiveness of Various Nesslerization Procedures in Protecting Against Turbidity Due to Creatinine, Uric Acid, and Urine

| *Procedure used in nesslerizing* | *Test substance* | *Reciprocal abscissal intercept (see text)* | |
|---|---|---|---|
| | | *Absence of $NH_3$* | *0.02 mg. $NH_3$-N* |
| Regular | Creatinine | 0.01 mg. | 0.10 mg. |
| Iodinated | Creatinine | 2.25 | 2.10 |
| $I_2$·KI | Creatinine | 0.13 | 0.20 |
| Gentzkow | Creatinine | 0.00 | 0.12 |
| NaOCl (Barrett) | Creatinine | 0.10 | .. |
| NaOCl (10x) | Creatinine | 1.10 | .. |
| Regular | Uric acid | 0.22 | 0.02 |
| Iodinated | Uric acid | 5.50 | 5.60 |
| Regular | Urine | .. | 0.01 ml. |
| Iodinated | Urine | .. | 0.32 ml. |

All the above values (except for Gentzkow) were determined by the two-stage nesslerization technic outlined in the text.

**Table 2.** Linearity and Sensitivity of Various Nesslerization Procedures

| *$NH_3$-N mg.* | *Optical density* | *K (O.D./mg. N)* | *Nesslerization method* |
|---|---|---|---|
| 0.05 mg. | 0.285 | 5.70 | Regular |
| 0.05 | 0.301 | 6.01 | Iodinated |
| 0.10 | 0.598 | 5.98 | Regular |
| 0.10 | 0.618 | 6.18 | Iodinated |
| 0.10 | 0.575 | 5.75 | Connerty |
| 0.10 | 0.485 | 4.85 | Barrett |
| 0.15 | 0.920 | 6.13 | Regular |
| 0.15 | 0.935 | 6.23 | Iodinated |
| 0.20 | 1.215 | 6.08 | Regular |
| 0.20 | 1.225 | 6.13 | Iodinated |
| 0.25 | 1.520 | 6.08 | Regular |
| 0.25 | 1.520 | 6.08 | Iodinated |

precipitated by alkaline Nessler's reagent, that it was not a reduction product of the reagent itself.

Various methods of deproteinization were tried in order to find one which would minimize this turbidity. Finally we tried a 5-minute period of heating in the presence of the zinc sulfate, with subsequent addition of barium hydroxide, hoping to denature resistant proteins, or to coprecipitate the interfering substance. This change was successful and is very similar to the procedure of Hoffman (6). It was incorporated into the final urea procedure (see Steps 4, 5, and 6 in *Procedures*).

### Urease Preparation and Stability

In the Karr (7) method for blood urea, it is essential to have a clear nonturbidogenic urease preparation. Since 1950, some difficulty has been

experienced in obtaining suitable alcohol extracts from jack-bean meal. Attempts to use crude commercial urease (8) were complicated by its rapid inactivation when dissolved in distilled water or in acetate buffer. For a time, neutralized, filtered gum acacia 2% was used to stabilize the enzyme solution. This addition was somewhat inconvenient although it did give good protection. Attempts to chelate the inactivating metals by ethylene diamine tetraäcetic acid (EDTA) also proved successful, and at present we are using the preparation shown in the list of reagents below. This urease solution maintains its original activity for at least 4 to 6 weeks in the cold, as determined by the colorimetric timing method of Van Slyke and Archibald (11).

### Comparison of Direct Nesslerization with Distillation-Nesslerization

To determine whether the new nesslerization technic did indeed prevent contribution by nonammonia chromogens to the color produced by ammonia upon nesslerization, a comparison was made of the amount of color produced by direct nesslerization and by nesslerization of the distillate of an identical aliquot. The distillation was accomplished *in vacuo* in an apparatus similar to the one described by Rinehart *et al.* (10). Blood and urine filtrates were prepared in the manner indicated in the prior section with and without the addition of a known amount of ammonia. These filtrates and their distillates were then nesslerized with the iodinated Nessler's two-stage technic and their optical density determined (against a distilled water optical blank set at 100). Table 3 gives the results of one such experiment.

**Table 3.** Comparison of Direct Nesslerization with Distillation-Nesslerization

| *Sample* | *Optical density* ($\times 10^3$) | |
|---|---|---|
| | *Directly nesslerized* | *Distilled nesslerized* |
| Blood filtrate | 007 | 006 |
| | 008 | 007 |
| | 007 | 007 |
| Blood filtrate plus 0.05 mg. $NH_3$-N | 309 | 309 |
| | 315 | 305 |
| | 313 | 309 |
| Urine filtrate (0.01 ml. urine) | 064 | 060 |
| | 060 | 058 |
| | 059 | 054 |
| Urine filtrate plus 0.05 mg. $NH_3$-N | 368 | 361 |
| | 359 | 361 |
| | 366 | 359 |
| Standard ammonium sulfate 0.05 mg. $NH_3$-N | 304 | 299 |
| | 299 | 298 |
| | 308 | 301 |

## DISCUSSION

Separation of the nesslerization procedure into two stages has made it possible to establish the optimum conditions of alkalinity and Nessler's salt concentration independently, so as to achieve maximum stability of the Nessler's-ammonia colloidal complex. Excess iodine can be dissolved in the neutral Nessler's salt solution as a convenient method of adding iodine to the sample to utilize the protective effect.

It has been shown that iodine, in quite effective concentration, does not adversely affect either the linearity or the sensitivity of the Nessler's reaction.

To ensure further against turbidity, a method of deproteinization has been suggested which combines the use of zinc sulfate, barium hydroxide, and heat.

These findings are each answers to specific problems associated with the determination of urea in blood and urine filtrates by direct nesslerization. It remains to combine them into a practical procedure. Undoubtedly, there are numerous convenient modifications possible. The approach used by us for the past 10 months is given below in detail.

### Urea in Blood and Urine Determined by Direct Nesslerization

Diluted blood or urine is treated with a buffered, EDTA-stabilized urease. A protein-free filtrate is prepared by the use of zinc sulfate, heat, and barium hydroxide. The filtrate is then nesslerized by the iodinated Nessler's solution using the two-stage technic.

#### Reagents

1. Ethylenediaminetetraäcetic acid (EDTA) (Versene, Sequestrene), 1% solution. Dissolve by adjusting to *p*H 6.8 with 2.5N NaOH.

2. Urease solution 0.5% in 1% (*p*H 6.8) EDTA. Keep cold.

3. Acetate buffer: Dissolve 100 Gm. sodium acetate ($3H_2O$) and 1.1 ml. of glacial acetic acid in water, dilute to 500 ml.

4. Urease-buffer mixture: Add 2 ml. urease solution and 2 ml. acetate buffer to 6 ml. $H_2O$ and mix. Prepare on the day of use.

5. Deproteinizing reagents: Barium hydroxide 0.06N; zinc sulfate, 1%. Titrate the zinc sulfate against the barium hydroxide by the procedure outlined by Nelson (12) or Somogyi (13).

6. Standard urea solution, stock: Dissolve 0.4287 Gm. pure dry urea in water and dilute to 1 liter, mix. Add two drops of toluene. This solution contains 20 mg. urea nitrogen per 100 ml.

7. Urea standard, working: Dilute 5.00 ml. of stock standard urea

solution to 100 ml. with distilled water and mix. Five milliliters contains 0.05 mg. urea nitrogen.

8. Ammonium sulfate, stock standard: Weigh out 0.9434 Gm. pure dry ammonium sulfate, dissolve in water and add one drop of conc. $H_2SO_4$. Dilute to 1 liter and mix. This solution contains 20 mg. ammonia nitrogen /100 ml.

9. Ammonium sulfate, working standard: Dilute 5.00 ml. of stock standard ammonium sulfate to 100 ml. with water, and mix. Five milliliters contains 0.05 mg. ammonia nitrogen.

10. Nessler's Stock Salt Solution (Koch-McMeekin).

11. Iodinated Nessler's salt solution: To 100 ml. of Nessler's Stock Salt Solution add 1.0 Gm. $I_2$ solid. Mix. Allow to stand until dissolved, mixing occasionally. Solution of the crystals may require several days. If necessary, the solution may be used before the iodine is completely dissolved.

12. 2.5N sodium hydroxide.

### Procedure

1. To 5.00 ml. distilled water in a test tube, add by multiple rinsing, 0.200 ml. of blood from a pipet calibrated to contain. Mix.

2. In addition to the blood tube, prepare similar tubes containing: (a) 5.00 ml. distilled water (reagent blank); (b) 5.00 ml. working urea standard; (c) 5.00 ml. ammonium sulfate working standard; (d) 1 to 5 ml. of a 1:200 dilution of urine (the exact amount depending on the urea concentration) made up to 5.00 ml. with water.

3. To each tube add 1.0 ml. urease-buffer mixture. Mix. Incubate in a water bath at 50° for 10 minutes.

4. Add to each tube 2.00 ml. 1% zinc sulfate. Mix.

5. Immerse tubes in a boiling water bath for 5 minutes.

6. Remove and cool. Add 2.00 ml. 0.06N barium hydroxide. Mix by vigorous shaking. Centrifuge.

7. Transfer 5.00-ml. aliquots of the supernatant (by means of a cotton-tipped pipet or after filtering) to matched photometer tubes.

8. To each tube add 1.0 ml. iodinated Nessler's salt solution. Mix. Then add rapidly (blow in or use a syringe pipet) 1.0 ml. of 2.5N NaOH. Mix each tube immediately after alkalinizing.

9. Allow to stand 5 minutes, read within an hour against the reagent blank set at 100, using a 515 mμ filter.

10. If the absorbance is above 90 or below 10, repeat, using more or less of the sample or filtrate.

### Calculations

Blood: $C_u = (D_u/D_s) \cdot 25.5$ = mg./100 ml. blood.
Urine: $C_u = (D_u/D_s) \cdot 5 \cdot V_{total}$ = mg./total volume of urine.

### Notes

1. 0.06N, NaOH, may be used instead of $Ba(OH)_2$, with only minute and occasional appearance of turbidities in the final solution.

2. Addition of iodinated Nessler's salt and 2.5N NaOH should be carried out at room temperature.

3. Urease is protected from heavy metal poisons by EDTA, blood proteins, and some other substances, but in urine dilutions and in pure urea solutions, not enough protection is present. Hence, care should be taken to keep nesslerization tubes (which contain mercury) separate from those used for incubation with urease.

4. The use of iodinated Nessler's salt solution prevents the reaction of such reducing substances as creatinine and uric acid with the alkaline Nessler's reagent.

5. Heating of the solutions in the presence of zinc sulfate is necessary to ensure complete removal of "protein" by the deproteinizing reagents.

6. The reproducibility of the color depends on rapid mixing when the alkali is added in the second stage of nesslerization.

7. Obviously, the sum of urine urea nitrogen and urine ammonia nitrogen is determined by this procedure. If a correction for the urine ammonia is desired, add the zinc sulfate before the urease, heat in the boiling water bath immediately, cool, and proceed as above.

8. If greater photometric accuracy in the low normal range is desired, use 5.00 ml. of a 1:10 Folin-Wu blood filtrate and proceed as directed. This then will result in a 0.25 ml. aliquot and $C_u = (D_u/D_s) \cdot 10$ = mg. urea nitrogen per 100 ml. blood.

### Urine Ammonia

Proceed as above with the following changes:

1. Use a 1:100 dilution of urine.
2. Omit the addition of urease-buffer (step 3)
3. Omit the urea standard; use the ammonium sulfate standard.

Calculation: Using 5.00 ml. of a 1:100 dilution
$C_u = (D_u/D_s) \cdot 100$ = mg. ammonia nitrogen per 100 ml. urine

### Stability of Urea Values Obtained by the New Procedure

Most of the published procedures for the photometric determination of blood and/or urine urea require that the optical measurement be made

**Table 4.** STABILITY OF RESULTS OBTAINED BY THE NEW NESSLERIZATION PROCEDURE

| *Specimen no.* | *Optical density* × $10^3$ | | *Urea value* | | *% change* | |
|---|---|---|---|---|---|---|
| | *10 min.* | *3 hr.* | *10 min.* | *3 hr.* | *O.D.* | *Urea* |
| 307 | 092 | 101 | 16.7 | 16.5 | +10.0 | −1.5 |
| 308 | 071 | 071 | 12.9 | 11.6 | 0.0 | −10.0 |
| 309 | 067 | 071 | 12.2 | 11.6 | +6.0 | −5.0 |
| 329 | 209 | 233 | 38.0 | 38.1 | +11.0 | +0.2 |
| 330 | 066 | 076 | 12.0 | 12.4 | +11.5 | +3.0 |
| 331 | 161 | 180 | 29.2 | 29.4 | +11.8 | +0.5 |
| 332 | 092 | 097 | 16.7 | 15.8 | +5.2 | −5.5 |
| 333 | 085 | 086 | 15.4 | 14.1 | +1.0 | −8.5 |
| 334 | 120 | 131 | 21.8 | 21.4 | +9.0 | −1.7 |
| 335 | 104 | 114 | 18.9 | 18.6 | +9.8 | −1.5 |
| 336 | 089 | 097 | 16.1 | 15.9 | +8.9 | −1.4 |
| 337 | 060 | 058 | 10.9 | 9.5 | −1.6 | −13.0 |
| 338 | 240 | 268 | 43.5 | 43.8 | +11.6 | +0.7 |
| 339 | 108 | 119 | 19.6 | 19.5 | +10.1 | −0.5 |
| 340 | 046 | 051 | 8.4 | 8.3 | +11.0 | −1.0 |
| 341 | 159 | 167 | 28.8 | 27.3 | +5.0 | −5.0 |
| $St_1$ | 138 | 152 | .. | .. | +10.1 | .. |
| $St_2$ | 143 | 160 | .. | .. | +11.9 | .. |
| $St_{average}$ | 140.5 | 156 | .. | .. | +11.0 | .. |

"within 2 minutes" or "rapidly before turbidity appears." This haste is inconvenient for large series. In order to demonstrate the stability of the results obtained by the new procedure, readings were made of a number of blood urea determinations, at 10 minutes and again at 3 hours after color development. The results are shown in Table 4. In the last two columns are given the per cent change in optical density values and in the calculated urea values between 10 minutes and 3 hours. Of course, both unknowns and standards have increased in optical density during this period and the blood urea values have been calculated with reference to the appropriate standard data.

The 3-hour values show a tendency to give somewhat lower values (−3.4 per cent on the average) than the 10-minute values. Nine of the sixteen 3-hour values for urea are within 2% of the 10-minute values.

## SUMMARY

1. The sources of turbidity in directly nesslerized blood and urine filtrates have been discussed and a new method of nesslerization has been presented which eliminates turbidities. The new procedure has been compared in a quantitative manner with several methods.

2. A procedure for the determination of blood and urine urea and for urine ammonia has been presented, incorporating the new nesslerization technic.

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# Urinary Peptide Excretion in the Burned Patient

*Bernard Balikov,* Robert A. Castello,† and Eloy R. Lozano*

PRONOUNCED CHANGES IN NITROGEN METABOLISM are known to occur as the result of a serious burn (1–8). Although considerable information is available on these changes, little attention has been given to the quantitative excretion of peptides in the urine of the burned patient. Such an assay has interesting theoretical possibilities. The nitrogenous products in the urine come from ingested food and body tissues. Since it is unlikely that urinary peptide excretion is direct from digested food (9, 10), it was felt that they probably originate in body tissues. Thus, liberation of peptides may be a reflection of tissue destruction or cellular disorganization, and this in turn could conceivably be a reflection of a major factor in the physiology of a burned patient.

In this report the urinary peptide loss will be correlated with the clinical estimate of the seriously burned patient. In so doing we will attempt to show how this relatively simple determination may be used to provide important information concerning the clinical condition of such a patient.

## MATERIAL AND METHODS

Studies were made on 38 patients admitted to the Surgical Research Unit for the treatment of burns. These burns ranged from minimal to lethal, and 16 of the 38 did not survive. Since normal values are not available on children (11), it was not possible to adequately interpret the

From the Biochemistry Section of the Surgical Research Unit, Brooke Army Medical Center, Fort Sam Houston, Tex.

* Present address: Department of Metabolism, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D. C.

† Present address: School of Pharmacy, University of Connecticut, Storrs, Conn.

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results on those who were burned. Thus, the lower age limit of patients dealt with in this report was held to 17 years.

Therapy was essentially in accordance with that given by Reiss and associates (12). During the first several days following a burn, administration of fluids is intensive to ensure a normal blood pressure. This is of importance to the present study in that it encourages adequate urine formation, since an analysis of urine would be of little value in a patient with near renal failure.

The Burn Index (13) was used to define the over-all seriousness of a burn with both second- and third-degree involvement. This index weights a second-degree burn as being one half as serious a clinical entity as a third-degree burn. Estimates of the per cent of body area involved and the degree of burn were usually averaged from the independent observations of at least two surgeons experienced in the handling of burned patients.

Urinary peptides were assayed by the biuret method of Balikov and Castello (11). Analyses were done on aliquots of 24-hour collections (8:00 A.M. to 8:00 A.M.) and are reported in terms of glutathione (GSH) equivalents. Normal values for adults are from 1.1 to 4.7 Gm. of peptide/24 hours as GSH. Specimens that were not assayed on the day of arrival (as on week-ends) were stored in a refrigerator. No samples were stored for more than 3 days.

Clinical evaluations as in diagnosing septicemia or excessive wound purulence were made available through the generous cooperation of the Bacteriology Section and the surgeons of the Clinical Ward of the Surgical Research Unit.

## RESULTS

Figure 1 illustrates the trend from elevated peptide excretions following the burn injury to normal values as the patient recovers. Each dot represents the average excretion for a 10-day period on a patient who survived. When two successive averages were normal, and the patient was considered clinically well, no further values were plotted. Results on 19 seriously burned patients (Burn Index 15 to 51) are represented. By the 70- to 80-day period only 1 patient (J. W., Burn Index 44) still had an average abnormal peptide excretion. With one exception, this patient had the most serious burn of those who lived. In no case was an abnormal value obtained on a patient who was completely healed. Two values seem exceptionally high for their period; *viz.*, 23.8 in the 40- to 50-day period and 14.9 in the 60- to 70-day period. In the first instance, the patient was receiving protein hydrolysate, orally, and in the second instance, the pa-

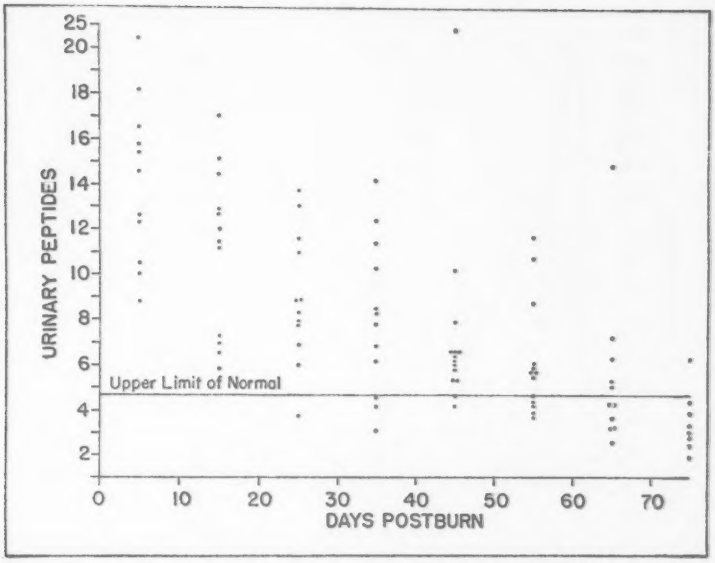


**Fig. 1.** The correlation between the urinary peptide excretion and days postburn on patients who recovered. Each dot represents the average excretion within its 10-day period. Normal values were not plotted after two successive averages were normal, and the patient was considered clinically well. (Urinary peptides are in terms of Gm. per 24 hours as GSH.)

tient had a septic arthritic condition and incipient congestive heart failure in addition to her burn. These were exceptional circumstances which could conceivably have an effect on peptide excretion.

The daily pattern of peptide excretion is shown in Fig. 2 for C. A. with a Burn Index of 15, and J. W. with a Burn Index of 44, both of whom recovered. These patients had relatively uncomplicated recoveries and so were deemed most suitable to illustrate the basic differences between mild and severe burns on survivors. It can be noted that some of the values in Fig. 2 are considerably higher than any values in Fig. 1. This is due to a phenomenon which can be termed "rhythmic spiking." Such spikes lasted only a few days and then subsided. When values are combined over a 10-day period as in Fig. 1, it is apparent that these spikes are masked in the over-all average. Figure 2 also illustrates three characteristics of the more serious burns: (1) higher spikes, (2) increased frequency of spiking, and (3) a longer time before normal values are obtained.

Figure 3 illustrates the daily pattern of peptide excretion for G. M.

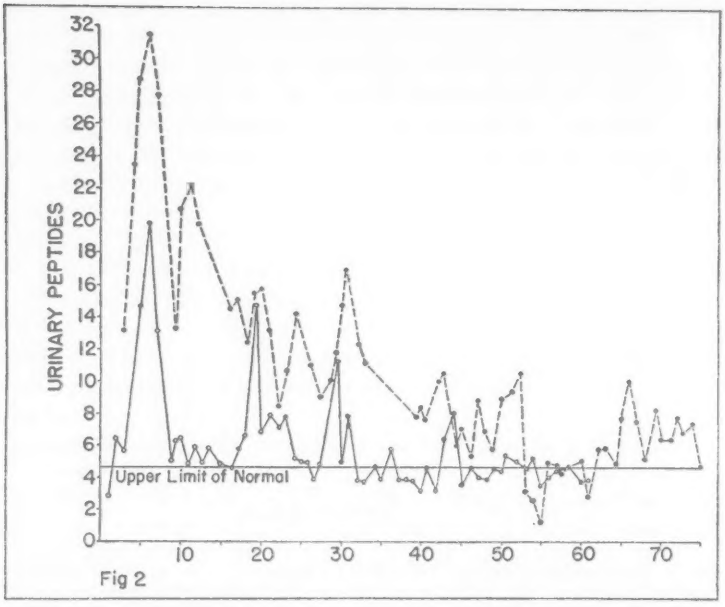


**Fig. 2.** The circles connected by a solid line represent the daily urinary peptide excretion pattern of C. A. who had a Burn Index of 15. The dots connected by a dash line represent this pattern for J. W. who had a Burn Index of 44. (Urinary peptides are in terms of Gm. per 24 hours as GSH.)

with a Burn Index of 38, and F. E. D. with a Burn Index of 47, both of whom died. Although in patients who recover, the highest spike is usually the initial spike reached within approximately 10 days postburn (Fig. 2), subsequent spikes reaching successively higher levels have been characteristic of patients whose condition was deteriorating.

In the line graphs of both Figs. 2 and 3 there are areas where no daily values are shown, as for example in Fig. 2 between days 33 and 39 on the dash line. Results were unavailable for several reasons, one of which was the formation of a yellow precipitate during the analytic procedure. This precipitate apparently takes up most of the copper of the biuret reagent since, when centrifuged, the supernatant is almost colorless. It is believed to be a cuprous oxide produced by a reducing substance, such as glucose (11). At other times, results were unavailable due to the presence of preservatives which were added in preparation of the urine for other analyses and whose effect on the peptide assay was unknown.

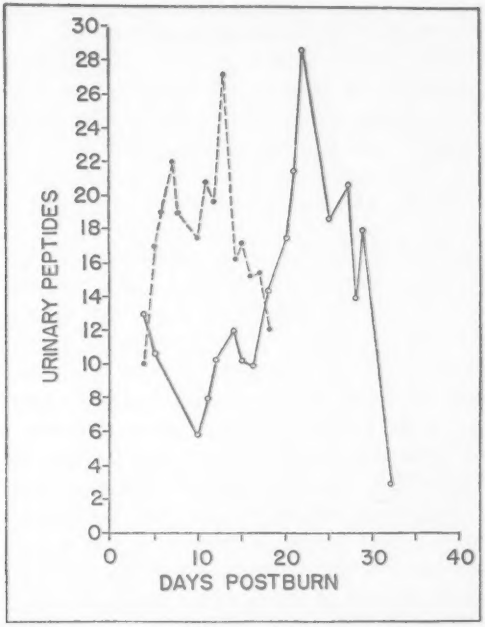


**Fig. 3.** The circles connected by a solid line represent the daily urinary peptide excretion pattern of G. M. who had a Burn Index of 38, and who died on his thirty-third postburn day. The dots connected by a dash line represent this pattern for F. E. D. who had a Burn Index of 47 and who died on his twentieth postburn day. (Urinary peptides are in terms of Gm. per 24 hours as GSH.)

### Associated Conditions and Therapy

An increased peptide excretion was found to accompany the onset of septicemia. Fourteen patients in this study were diagnosed as having septicemia sometime during their course of treatment. Of these, 8 had peptide values available on the day preceding and the day of onset of septicemia. The average increase of urinary peptide excretion in this 24-hour period was 7.5 Gm. as GSH. Increases ranged from 3.1 on a patient (M. L.) with a positive blood culture but without a spiking temperature, chills, or prostration, to 14.7 on a patient (J. C.) who died 4 days later. Patient M. L. had a temperature up to 104.6° 8 days after the positive blood culture was found. His peptide excretion increased 9.0 Gm. from the day before.

Table 1 illustrates the moderate increase in peptide excretion to be expected as the result of an operative procedure such as grafting. ("Post-

**Table 1.** The Effect of Grafting on Peptide Excretion
Postgraft excretion minus pregraft excretion

| *Burn Index* | *Days Postburn* | |
|---|---|---|
| | *0 to 30* | *Over 30* |
| | Average difference in Gm. of peptide/24 hrs. as GSH. | |
| 15–24 | 3.6 | 1.8 |
| 25–34 | 4.6 | 2.4 |
| Over 35 | 3.8 | 2.7 |

graft" excretion refers to the day of the operation and "pregraft" excretion refers to the day before the operation.) There appears to be a trend for this increase to be of lesser magnitude if the operation occurs after 30 days postburn. In fact, 6 of the 57 values used to compile these averages showed a decrease in peptide excretion all occurring in the period after 30 days postburn. This may be due to smaller grafting procedures or an increased ability of the patient to cope with an operative procedure, or both, since most patients are well on their way to recovery by this time. No clear-cut relation between the extent of burn and increase in peptide excretion resulting from an operative procedure is evident.

When they were almost healed, 2 patients were given growth hormone (Armour's). A course of therapy consisted of 50 mg. intramuscularly every 6 hours for 5 days. A total of seven such courses of therapy were given. In all but one instance, there was an increase in peptide excretion with maximum values usually exhibited 3 to 5 days after the onset of therapy. These maximum values showed an average increase of 4.0 Gm. over the excretion the day before therapy was begun. This effect of growth hormone is difficult to interpret. There was a concomitant weight loss of about 4 pounds over the 5-day period of medication in each of the six instances with elevated peptide excretion. In the one case of diminished excretion, the patient gained about 5 pounds during the course of therapy. Members of the Surgical Research Unit presently investigating the effect of growth hormone on the burned patient, are of the opinion that its effect on weight and nitrogen balance depends, at least in part, on nitrogen intake which was uncontrolled in this study.

Two patients received protein hydrolysate[1] intravenously. This resulted in an increase in peptide excretion of from 5 to 10 Gm. as GSH per liter of hydrolysate. This is not surprising since the molecular size of most of the fractions would permit easy passage through the kidney. It has been mentioned by Christensen (9) that there is large urinary wastage upon injection of peptides.

[1] Five per cent bovine plasma digested to free amino acids and polypeptides of which at least 50 per cent of the total nitrogen was present as free $\alpha$-amino nitrogen.

Tarry and bloody stools indicative of gastrointestinal bleeding were noted on 4 patients. The day on which bleeding was noted showed a slight increase in peptide excretion (average 2.0 Gm.) compared with the day before. However, maximum values were usually reached 2 to 3 days later, at which time the increase over the day before bleeding began averaged 12.6 Gm. The range of maximum increases varied considerably from 3.4 on a patient with possible kidney damage to 32.1 on a patient who died 4 days later with a diagnosis of bleeding duodenal ulcer, peritonitis, and hemorrhage of the pancreatic duodenal artery.

Excessive wound purulence uncomplicated by septicemia or bloody stools is usually (11 of 12 instances) accompanied by a measurable increase in peptide excretion. Since the extent of wound purulence is usually noted at a dressing change, increases are measured from the day of the last dressing change at which purulence was not noted to the day on which purulence is noted. The average increase in this period was 3.0 Gm. The maximum increase during this period was 3.5 Gm.

Several other conditions appeared to be associated with increased urinary peptide excretion but occurred too infrequently to generalize on relationship. They were: (1) 2 patients with low plasma calcium levels; (2) 2 patients on heparin therapy for the treatment of thrombosis; (3) 2 patients with tachycardia; (4) 1 patient who in addition to a bacteremia, developed a yeast growth in his blood; and (5) 1 patient who developed incipient congestive heart failure.

Temperature rises unaccompanied by septicemia, purulent wounds, etc., had no apparent effect on peptide excretion. An increase of 1° F. or more was accompanied by ten instances of increased and ten of decreased peptide excretion. Limiting the correlation to temperature increases of at least 2° F. showed six instances of increased and five of decreased excretion.

Nine healthy, normal male soldiers were put on bed rest and fed varying amounts of protein as part of a concurrent nitrogen balance study. The urinary excretion of peptides was followed on these men. Their intake varied from 5.6 to 337 Gm. of protein in any particular 24-hour period. The correlation between this varied intake and the urinary peptide excretion was determined by the regression analysis technic described by Snedecor (14). Based on $n = 53$, a $t$ value of 1.26 was obtained which is equivalent to a $p$ value between 0.2 and 0.3. There is apparently no significant relation between protein ingestion and peptide excretion in the normal person at bed rest.

A correlation was sought between protein ingestion and the urinary peptide excretion in a burned patient. J. W. with a Burn Index of 44, was

**Table 2.** THE RELATION BETWEEN PROTEIN INTAKE AND URINARY PEPTIDES IN A SEVERELY BURNED PATIENT

| *Postburn day* | *Protein intake* (Gm./24 hr.) | *Urinary peptides* (Gm./24 hr. as GSH) |
|---|---|---|
| 6 | 39.4 | 31.4 |
| 7 | 44.2 | 27.7 |
| 8 | 44.2 | 18 |
| 9 | 44.2 | 13.4 |
| 10 | 44.2 | 20.6 |
| 11 | 16.9 | 22.2 |
| 12 | 16.9 | 19.7 |

placed on controlled protein intakes and was studied from his sixth through twelfth postburn day. The results shown in Table 2 make it unnecessary to confirm by statistical analysis the lack of correlation between protein intake and peptide excretion in this severely burned patient.

## DISCUSSION

It must be recognized that this method for assay of urinary peptides yields results in terms of GSH equivalents and not as the absolute amount of peptide or peptide nitrogen (11). Thus, for example, an excretion of 20 Gm./24 hours as GSH does not necessarily mean that the equivalent of 20 Gm. of protein has been excreted since the relation between these two values has not yet been elucidated.

The increase in peptide excretion which accompanies septicemia was always noted with the first signs (15) of this condition whether bacteriologic or clinical. In 4 of the 8 cases studied, the urinary peptide increased the day before the blood showing the positive culture was drawn. It is therefore suggested that a sudden unexplained increase in peptide excretion stimulate concern over the possibility of septicemia being present.

A serious burn is rarely an uncomplicated insult. It is always accompanied by surgical procedures. In addition, septicemia, wound purulence, gastrointestinal ulcers, etc., are not uncommon. All these complicating phenomena give rise to a "hyperpeptiduria" unless, of course, there is concomitant renal failure. On the other hand, oral protein intake and uncomplicated temperature fluctuations have no effect on the excretion of urinary peptide. This information is consistent with the theory that the source of the peptides in the urine is endogenous and that "hyperpeptiduria" is a reflection of tissue destruction or cellular disorganization. The assay of urinary peptides may thus serve as an objective laboratory aid to the physician in his evaluation of the condition of a burned patient.

## SUMMARY

The pattern of peptiduria in serious burns has been presented. This pattern has been found to vary, depending upon whether the patient's condition deteriorates or improves. The changes in this pattern resulting from various complications often seen in an extensive burn have been noted.

Evidence has been presented supporting the theory of the endogenous origin of urinary peptides.


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# Micro Determination of Cholesterol by Use of 0.04 Ml. of Blood Serum

## Adapted from the Method of Sperry and Webb

*Leora S. Galloway, Patricia W. Nielson, Ethelwyn B. Wilcox, and Edith M. Lantz*

THE USE OF MICRO METHODS has enabled investigators to determine several constituents on one sample of blood obtained by simple finger-tip puncture. Only 0.005 to 0.05 ml. of serum is needed for the analyses of vitamins and certain other constituents (1). A method is herein reported for determining free and total cholesterol through the use of a similar volume of serum (0.04 ml.). This micro procedure has been adapted from the macro method of Sperry and Webb (2) which was a modification of the so-called "micro" method developed in 1934 by Schoenheimer and Sperry (3). This "micro" method used 0.2 ml. of serum for the free and combined cholesterol analyses. However, the use of only 0.04 ml. of serum would leave enough serum from one sample of finger-tip blood for other determinations in addition to the cholesterol analyses. The results obtained from the micro technic have been compared with results obtained from the macro method of Sperry and Webb (2).

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From the Utah Agricultural Experiment Station, Logan, Utah, and the New Mexico Agricultural Experiment Station, State College, N. M.

Present addresses: (L. S. G. and E. B. W.) Utah State Agricultural College, Logan, Utah; (P. W. N.) 114 South LeBaron St., Mesa, Arizona; (E. M. L.) New Mexico A. & M. College, State College, N. M.

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The micro method described in this report has been used in the analyses of serum from finger-tip blood of 753 school children at the Utah station and of 330 school children at the New Mexico station.

## EXPERIMENTAL METHODS

The blood serums used for comparison of the micro and macro methods were obtained from 8 healthy young women and from 21 rats on various types of diets. The reagents used were the same as listed by Sperry and Webb (2).

### EQUIPMENT

1. 1 ml. volumetric flasks with stoppers.
2. 5, 10, 30, 40, 50, 100, and 200 λ pipettes.
3. 1 ml. syringe with needle and automatic syringe holder.
4. 1 ml. and 3 ml. test tubes with suitable racks to hold them.
5. Refrigerated centifuge or centrifuge that can be placed in refrigerator.
6. Wide-mouth pint preserving jars with lids, containing 1½ inches of sand.
7. Beckman Spectophotometer with micro attachment as described by Bessey (1).
8. Mixing device—Handee grinder (Chicago Wheel Mfg. Co.), used to buzz the solutions.

### PROCEDURE

The same day the blood was collected 40 λ of blood serum were measured into 1 ml. volumetric flasks, stoppered, and frozen until ready for analyses. The 40 λ gave enough serum for duplicate determinations of both free and total cholesterol.

To extract the serum, 0.4 ml. of acetone-ethanol was added with gentle force directly into the serum in the 1 ml. volumetric flasks with the 1 ml. automatic syringe. This helped to prevent clumping of precipitate. Each flask was buzzed immediately until the precipitate was divided into fine particles. The solvent was brought to a boil by placing each flask in a beaker of boiling water. The flask was cooled to room temperature at once by placing it in cold water, and the solution was made up to 1 ml. volume with acetone-ethanol. The solution was centrifuged at 2500 rpm for 20 minutes so that the precipitate was packed tightly against the bottom of the flask. The filtrate was poured off carefully into a 10 x 75 mm. test tube and aliquots were pipetted

immediately for free and total cholesterol. (The aliquots may also be pipetted directly from the flask).

### FREE-CHOLESTEROL DETERMINATION

For the free cholesterol determination, 200 λ of the filtrate were pipetted into each of two 1 ml. test tubes. Five λ of 10% acetic acid and 100 λ of digitonin solutions were added to each tube to precipitate the free cholesterol as cholesterol digitonide. Each tube was buzzed well, fitted with rubber cap, placed in covered pint jar, and allowed to stand over night at room temperature. A blank containing 200 λ of the acetone-ethanol reagent was run at the same time.

The tube was tapped well with the finger to free any precipitate which adhered to the wall near the surface of the liquid. Each tube was centrifuged for 20 to 30 minutes. By means of slow suction the supernatant solution was drawn off with a fine-tipped transfer pipet. Careful separation may be facilitated by the use of a Bausch and Lomb lighted magnifying lens. The wall of the tube was washed with 200 λ of acetone-ether from an automatic 1 ml. syringe with needle. The remainder of the washing was done as in the macro method (2). The precipitate was dried by placing the tube in a small beaker of hot water for a few minutes. The sample may be stored several days at this stage.

### TOTAL-CHOLESTEROL DETERMINATION

For the determination of total cholesterol, 100 λ of the filtrate were pipetted in duplicate into 1 ml. test tubes, to each of which was added 5 λ of 33% KOH solution. Each was buzzed until it was well emulsified, fitted with rubber cap, and placed in a pint jar in an incubator (37° to 40°) for 30 minutes. A blank containing 100 λ of the acetone-ethanol reagent was given the same treatment.

After each tube had been cooled to room temperature, 120 λ of acetone-ethanol were added to make approximately 200 λ of solution. Five λ of phenolphthalein and 30 λ of 10% acetic acid solutions were added. If the sample was not colorless, 5 λ more of the acetic acid solution were added so that the solution was slightly acid in reaction, 100 λ of digitonin solution were added, and each tube was buzzed well. These tubes were stored in the same manner as tubes of free cholesterol.

The procedure for washing each of the total cholesterol samples was the same as that described for free cholesterol, except that the ether washing was done only once.

### COLOR DEVELOPMENT

In the development of the color, the cholesterol digitonide was dried by the procedure used by Sperry and Webb (2). Fifty λ of pure glacial acetic acid were added while the tube was in the hot sand bath. After 2 or 3 minutes the tube was rotated slightly on its side to make sure the acid came in contact with all the precipitate. The tube was tapped with the finger about 5 times and placed in a wire rack. This process of adding and mixing was continued until all the tubes were in the rack in the order of reading. A check tube containing 50 λ of glacial acetic acid was prepared to use in setting the Beckman Spectrophotometer at zero. Three tubes of standard solution (5 μg. of cholesterol per 50 λ) were equally spaced in the wire rack, one at the beginning, one in the middle, and one at the end of the series of 44 tubes, and the rack of tubes was placed in a 28° water bath.

The acetic anhydride-sulfuric acid mixture was prepared as in the macro method. The solution was allowed to cool 10 minutes before using.

Since the time of color development needed to be the same for all samples, 100 λ of the acetic anhydride-sulfuric acid reagent were added to each tube in the water bath in groups of 3 every 2 or 3 minutes. Each sample was read on the instrument exactly 30 minutes after the reagent had been added.

The procedure for reading the color on the Beckman Spectrophotometer was as follows: The instrument was set at a slit width of 0.44 mm., wave length at 625 mμ, and no filter was used. The cuvets were not washed between readings, but all liquid was drawn out with a transfer pipet by the use of suction. One cuvet only was used for check solution, one only for reagent blanks, one only for the standard, and 3 other cuvets for samples.

The calculation (4) is as follows:

$$\text{Mg. cholesterol per 100 ml. of serum} = \frac{\left\{\begin{array}{l}\text{density of unkown} - \\ \text{density of reagent blank}\end{array}\right\}}{\text{density of standard}} \times \text{mg. cholesterol in standard} \times \frac{100}{\text{volume of unknown}}$$

## RESULTS AND DISCUSSION

In the development of the micro method for cholesterol many different variations were tried, such as varying the amount of KOH used in the saponification; stirring rods vs. no stirring rods; different

volumes in which the color was developed; various lengths of time for centrifuging; and centrifuging at room temperature in comparison to refrigeration temperature.

The volume of 50 λ glacial acetic acid to 100 λ acetic anhydride-sulfuric acid reagent gave a much better reading than a volume of twice that amount for each reagent. It also gave sufficient solution to read in the micro cuvets.

The addition of acetone-ethanol to the blood serum gave a much finer precipitate than when the reverse was done, especially when acetone-ethanol was added with slight force directly into the serum.

The percentage recovery of cholesterol in acetone-ethanol was 96.8 for this micro method.

A comparison of values obtained on human serum by the micro and macro methods is given in Table 1. Average values for the micro method were 55.1 mg. and 214 mg. per 100 ml. of serum for free and total cholesterol as compared to 55.8 and 216, respectively, for the macro method. This gave a difference between the two methods of 2.4 per cent for the free and 1.1 for the total cholesterol average values.

The results obtained on the rat serum are presented in Table 2. Average values for the micro method for free and total cholesterol were 24.4 mg. and 120 mg. per 100 ml. of serum as compared to 21.3 and 119 mg., respectively, for the macro method. The percentage difference between the micro and macro values was quite great for

**Table 1.** COMPARISON OF CHOLESTEROL VALUES FOR HUMAN SERUM

| | *Micro method* | | | *Macro method* | | |
|---|---|---|---|---|---|---|
| *Sample no.* | *Free cholesterol (mg./100 ml.)* | *Total cholesterol (mg./100 ml.)* | *Ratio* | *Free cholesterol (mg./100 ml.)* | *Total cholesterol (mg./100 ml.)* | *Ratio* |
| 1 | 49.6 | 189.0 | 0.262 | 48.2 | 188.8 | 0.255 |
| 2 | 69.0 | 272.4 | 0.254 | 69.2 | 269.0 | 0.257 |
| 3 | 54.2 | 213.1 | 0.254 | 51.6 | 211.3 | 0.244 |
| 4 | 48.0 | 185.8 | 0.258 | 45.1 | 190.0 | 0.237 |
| 5 | 58.7 | 211.4 | 0.278 | 54.9 | 219.6 | 0.250 |
| 6 | 77.7 | 296.4 | 0.262 | 77.8 | 303.2 | 0.255 |
| 7 | 52.0 | 205.7 | 0.253 | 50.1 | 213.0 | 0.235 |
| 8 | 31.4 | 138.0 | 0.228 | 33.3 | 136.1 | 0.244 |
| Avg. | 55.1 | 214.0 | 0.256 | 53.8 | 216.4 | 0.249 |
| Range | 31.4-77.7 | 138.0-296.4 | 0.228-0.278 | 33.3-77.8 | 136.1-303.2 | 0.235-0.257 |

**Table 2.** COMPARISON OF CHOLESTEROL VALUES FOR RAT SERUM

| Sample no. | *Micro method* | | | *Macro method* | | |
|---|---|---|---|---|---|---|
| | *Free cholesterol (mg./100 ml.)* | *Total cholesterol (mg./100 ml.)* | *Ratio* | *Free cholesterol (mg./100 ml.)* | *Total cholesterol (mg./100 ml.)* | *Ratio* |
| 1 | 15.0 | 71.6 | 0.210 | 13.6 | 63.0 | 0.215 |
| 2 | 21.4 | 83.6 | 0.255 | 16.0 | 76.0 | 0.211 |
| 3 | 22.2 | 86.7 | 0.256 | 19.2 | 82.0 | 0.234 |
| 4 | 20.4 | 99.0 | 0.207 | 20.3 | 91.3 | 0.222 |
| 5 | 18.2 | 79.3 | 0.231 | 16.6 | 73.0 | 0.227 |
| 6 | 38.2 | 173.4 | 0.220 | 33.9 | 176.0 | 0.193 |
| 7 | 27.7 | 132.8 | 0.208 | 24.7 | 136.9 | 0.180 |
| 8 | 19.9 | 94.8 | 0.209 | 17.9 | 99.7 | 0.180 |
| 9 | 30.7 | 149.9 | 0.205 | 29.0 | 142.8 | 0.203 |
| 10 | 19.6 | 105.3 | 0.186 | 16.8 | 93.4 | 0.180 |
| 11 | 48.2 | 219.0 | 0.220 | 43.2 | 204.8 | 0.211 |
| 12 | 47.8 | 200.4 | 0.238 | 40.1 | 201.0 | 0.200 |
| 13 | 19.8 | 115.6 | 0.171 | 16.0 | 115.6 | 0.138 |
| 14 | 18.8 | 124.1 | 0.151 | 18.2 | 134.2 | 0.136 |
| 15 | 17.3 | 97.6 | 0.177 | 11.8 | 91.2 | 0.129 |
| 16 | 13.1 | 88.6 | 0.148 | 9.6 | 93.2 | 0.103 |
| 17 | 23.7 | 113.6 | 0.209 | 19.2 | 114.4 | 0.168 |
| 18 | 22.6 | 124.3 | 0.182 | 19.4 | 126.4 | 0.153 |
| 19 | 20.6 | 120.2 | 0.171 | 17.8 | 127.5 | 0.140 |
| 20 | 23.0 | 134.8 | 0.171 | 18.7 | 141.0 | 0.133 |
| 21 | 25.2 | 103.5 | 0.243 | 25.6 | 111.4 | 0.230 |
| Avg. | 24.4 | 119.9 | 0.204 | 21.3 | 118.8 | 0.179 |
| Range | 13.1-48.2 | 71.6-219.0 | 0.148-0.256 | 9.6-43.2 | 63.0-204.8 | 0.103-0.234 |

some of the rat serum which had low values for free cholesterol but in every case the micro technic gave higher values.

In considering all factors involved, the micro method has several advantages over the macro method; namely, more samples can be handled in the same length of time, centrifuging is less of a problem because of smaller tubes, omission of stirring rods reduces the chance of error, and the expense of the reagents is reduced considerably. The micro method, if handled with great care as to technic, should give as reliable results as the macro method.

## SUMMARY

A micro method for cholesterol determination adapted from the macro method of Sperry and Webb is described with comparisons of

results obtained by the two methods. In general, the two methods gave similar results for free and total cholesterol.

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# Estimation of Total Cholesterol in Serum by a Micro Method

*K. J. Carpenter, A. Gotsis, and D. M. Hegsted*

ALBERS AND LOWRY (1) HAVE PUBLISHED a method for the estimation of cholesterol on a micro scale in animal tissue by fluorometry involving the reaction of extracted cholesterol dissolved in trichloroethane with acetic anhydride and sulfuric acid. The present paper describes a simple and tested use of this reaction for the estimation of total cholesterol in 0.02 ml. of serum after extraction by a standard procedure (2) and without use of special apparatus.

## EXPERIMENTAL

### APPARATUS

Farrand fluorometer, set to aperture size 6, with Corning filters 4010 and 5120 in the primary path (a thin holder to take the extra width being used) and filter 2424 in the secondary path (1).

### REAGENTS

1. Alcoholic KOH, freshly prepared from 6 ml. of 33% KOH and 94 ml. of redistilled absolute alcohol.
2. Petroleum ether, fraction boiling at 68°, redistilled.
3. "Trichloroethane-acetic anhydride," 5 parts of 1, 1, 2-trichloroethane (Eastman Kodak) used without redistillation and 1 part acetic anhydride. This reagent should be used within one hour after preparation.

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From the Department of Nutrition, Harvard School of Public Health, Boston, Mass.

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4. Sulfuric acid, reagent grade.

5. Cholesterol standard, 400 μg. cholesterol (recrystallized four times) per ml. of absolute alcohol.

6. Rhodamine B standard, .032 μg. Rhodamine B (Eastman Kodak) per ml. of $H_2O$.

## PROCEDURE

One milliliter of alcoholic KOH is measured into a 15 ml. glass-stoppered vial, and 0.02 ml. of serum is added with a hemoglobin pipet (with rinsing). The vial is stoppered and placed in a water bath at 37-40° for 55 minutes. After the vial has been cooled to room temperature, 4 ml. of petroleum ether and 1 ml. of water are added. The vial is shaken for 2 minutes, and the layers are allowed to settle. Two ml. of the supernatant (ether) layer are then transferred to a 125 x 15 mm. tube.

The solvent is evaporated from the tube by placing it in a water bath at approximately 60° and injecting an air stream from a nozzle placed in the top of the tube. The tube is allowed to cool to room temperature; 2 ml. of trichloroethane-acetic anhydride are added and the tube is shaken gently. After 15 minutes, 0.08 ml. of sulfuric acid is added with a 0.2 ml. pipet graduated in hundreths and the tube is shaken immediately.

After 15 to 60 minutes, approximately one-quarter of the contents is used to rinse out a 9 x 75 mm. fluorometer tube; the remainder is then tipped in, and the fluorescence is measured with the sensitivity control of the instrument set to give 50 per cent of "full deflection" with the Rhodamine B solution which is kept in a second tube. (This adjustment is checked before each "unknown" reading).

Two levels of cholesterol and a blank are run simultaneously through the entire analytical procedure including the extraction. Usually 0.10 and 0.20 ml. of standard cholesterol solution, each in triplicate, have been used. These amounts are measured into the alcoholic KOH with a 0.2 ml. pipet graduated in hundredths.[1] The blank consists of 1 ml. of KOH.

## CALCULATION RESULTS

The fluorometer readings from the standards and blank are plotted graphically against the initial volume of cholesterol standard used.

[1]Although graduated pipets have been used throughout, micro constriction pipets (3) are as satisfactory and convenient.

The volume corresponding to the reading obtained with a serum sample is read from the graph. This figure multiplied by 2000 gives the "total" cholesterol content of the serum in mg. per 100 ml. When sera contain so much cholesterol that readings exceed the top of the standard curve, 1 ml. in place of 2 ml. of the petroleum ether extract can be used. If this is still inadequate, then 2 ml. of extract can be diluted to 10 ml., an aliquot taken, and appropriate changes in the method of calculation made.

## RESULTS AND DISCUSSION

### RESPONSE TO CHOLESTEROL STANDARD

As expected (1), this was linear in the range indicated, provided that the incident light in the fluorometer was adjusted to its lowest level (i.e., aperture size 6). With the sensitivity adjusted so that the highest cholesterol standard (giving a final concentration of 15 $\mu$g./ml. in the fluorometer tube) gave a reading of approximately 90, the blank reading was usually in the range 6-9 after completion of the whole procedure. This might have been further reduced by redistillation of reagents (1).

### SPECIFICITY

The specificity of the fluorescence has already been studied (1). Of the other steroids giving fluorescence, none is expected to occur in serum in quantities which would yield misleading results. This is confirmed by the agreement of the results for sera with those obtained by the Abell method for which it has been demonstrated that not less than 99 per cent of the values obtained with sera are specifically due to cholesterol (2).

### CRITICAL VARIABLES

The results are not affected by a wide range of laboratory temperatures or by carrying out the determinations in sunlight. Obviously, any errors in pipetting (except for alcoholic KOH) will be reflected in changes in final serum concentrations. Similar dilution errors will follow from failure to prevent evaporation of petroleum ether before the aliquots are taken, or from incomplete evaporation in the water bath. The procedure can be interrupted just prior to evaporation of the petroleum ether (2).

The final addition of 0.08 ml. sulfuric acid is accomplished by using a 0.2 ml. graduated pipet. This step is particularly critical. In test

runs the addition of 0.07, 0.075, 0.085, and 0.09 ml. have given 94, 99, 103, and 105 per cent, respectively, of the fluorescence obtained with 0.08 ml. of acid.

### RESULTS WITH RAT SERA

Thirty-two serum samples with a range of 60 to 300 mg. per 100 ml. cholesterol were taken from female rats of approximately 250 Gm. weight that had been on experimental diets with and without cholesterol and methionine supplements for 6 weeks.[2] They were analyzed for total cholesterol by the micro method described with complete replication (i.e., with the replicate analyses carried through on different days and, therefore, with different standard curves), and also by the Abell method (2) in which duplicate aliquots of petroleum ether from a single extraction were used.

The individual results are presented in the form of a scatter diagram (Fig. 1). The over-all mean for the Abell determination on all the sera was 126 mg. per ml.; the micro method gave an over-all mean 3 mg. per 100 ml. lower, and the standard deviation of this difference for the individual sera was ±8 mg. per 100 ml. The standard deviation due to error within the micro method for a single value determined in duplicate (as calculated from the differences between replicates) was ±5 mg. per 100 ml.

### RESULTS WITH HUMAN SERA

A second similar trial was conducted with 43 samples of human serum (obtained during routine examination of apparently normal subjects) covering a range from 160 to 380 mg. per 100 ml. in their cholesterol content.

The results were similar to those obtained for rat sera. The over-all mean for the series was 253 mg. per 100 ml. by the Abell method, and 1 mg. per 100 ml. lower by the micro method. The standard deviation of the difference between the macro- and micro-method results for individual sera was 11 mg. per 100 ml. The standard deviation within the micro-method was ±4 mg. per 100 ml. for this series, when calculated in the same way as for the results with rat sera.

### ROUTINE APPLICATION

The findings of the special trials and the general results obtained during routine use in the laboratory indicate that the method is suit-

[2]Carpenter, K. J., Gotsis, A., and Hegsted, D. M., material in preparation for publication, 1956.

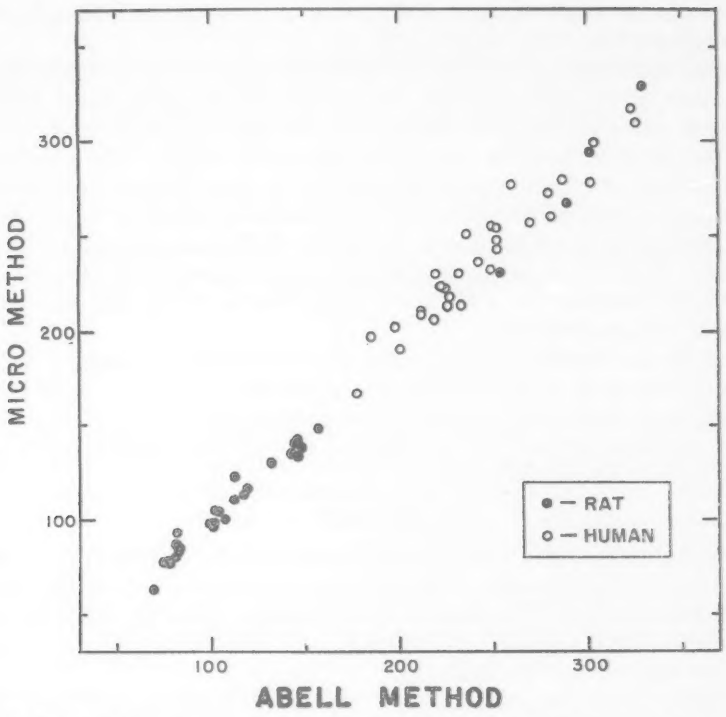


**Fig. 1.** Correlation diagram showing the results of estimation of total serum cholesterol in 32 samples each of rat and human serum by the Abell and micro methods.

able for the estimation of total serum cholesterol from both human subjects and experimental animals.

There seems to be no advantage in using quantities less than 0.02 ml. With lipemic sera, particularly, even shaking to break up clots might be inadequate to give good sampling with a lower volume. At the 0.02 ml. volume, the fluorescence developed is sufficiently intense so that regular apparatus and cuvets can be used. Modification of the colorimetric methods of cholesterol estimation for smaller samples has involved the use of micro equipment (4).

It has been confirmed that the fluorescence remains constant for the period from 15 to 60 minutes after the addition of the final reagent (sulfuric acid) (1), and this has allowed about 60 tubes to be carried

through the reagent stage and then read in one batch, even though the two stages take rather different times.

The method used routinely for collection of rat and mouse sera was to bleed 0.2 to 0.3 ml. from the tail into a 2 ml. centrifuge tube. After standing for an hour, this was centrifuged and 0.02 ml. aliquots were taken directly from the tube with hemoglobin pipets. The bleeding and taking of serum aliquots may have to be done the same day since storage overnight of whole blood significantly changed values for lipemic samples with cholesterol values of 1000 mg. per 100 ml. or more. However, it has proved possible for two people to bleed 60-70 rats and complete the estimations in duplicate, with a full standard curve, in a working day.

Since the adoption of this method, several thousand samples have been carried through without any apparent difficulties. Different batches of trichloroethane have given significantly different slopes to the standard curves, but the values obtained for check samples of serum have been unchanged.

## SUMMARY

A method has been described for the estimation of total cholesterol in 0.02 ml. of serum without the need for micro apparatus. It involves the measurement of the fluorescence developed after the addition of sulfuric acid to a solution of cholesterol in a mixture of 1,1,2-trichloroethane and acetic anhydride (1).

The results obtained with both human and rat sera have been in good agreement with those obtained with a standard colorimetric method (2), and the standard deviation for an estimation carried through in duplicate, owing to error within the method, has been ±4-5 mg. per 100 ml. under routine conditions.

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# Epinephrine and Norepinephrine Concentrations in Plasma of Humans and Rats

*Irving Gray and James G. Young*

With the Technical Assistance of James F. Keegan, Benjamin Mehlman, and Eldred W. Southerland

THE FLUORIMETRIC METHOD of Weil-Malherbe and Bone (1, 2), with minor modifications, has been applied successfully in this laboratory to the measurement of epinephrine and norepinephrine in the plasma of normal human subjects and rats subjected to traumatic injury. The measurement of these hormones in normal rats has been reported elsewhere (3).

This report describes the modifications (resulting from the use of materials readily available in this country) which have been made, the precision and accuracy of the method, using both human and rat plasma, proof of specificity of the method as applied to rat plasma catechol amines, and the measurement of plasma catechol amines in human subjects of various ages.

## MATERIALS AND METHODS

The technic reported in this paper differs from the method of Weil-Malherbe and Bone (1) in the following items:

*Instrument:* Photofluorometer (Farrand Optical Company).

*Filters:* All filters manufactured by Corning Glass Company.

Primary:

(1) No. 5113—one "standard" thickness and one one-half thickness.

(2) No. 3389—"standard" thickness.

From the Department of Biochemistry, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington 12, D. C.

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Secondary:

(1) No. 3384—"standard" thickness
(2) No. 5433—"standard" thickness
(3) No. 3486—"standard" thickness

Figures 1, 2, and 3 illustrate the spectral characteristics of the filter combinations used.

## REAGENTS

These were (a) ethylene diamine (Eastman Kodak, "white label"); (b) ethylene diamine dihydrochloride (Eastman Kodak, "white label"); (c) isobutanol (Merck, for fluorimetric use); (d) aluminum oxide, 80-200 mesh (alumina, adsorption, for chromatographic analysis, Fisher Scientific Company). Reagents were prepared according to the directions of Weil-Malherbe and Bone, except that the ethylene diamine was distilled through a 20 cm. Vigreaux column just before use, in order to obtain minimum fluorescent blanks. After distillation this reagent can be stored for several days in a glass-stoppered brown bottle.

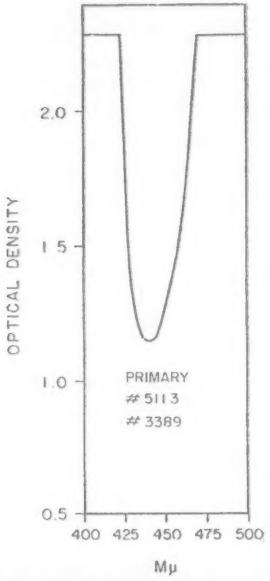



**Fig. 1.** Spectral characteristics of primary filter.

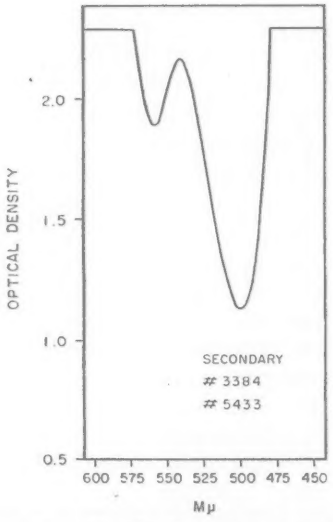
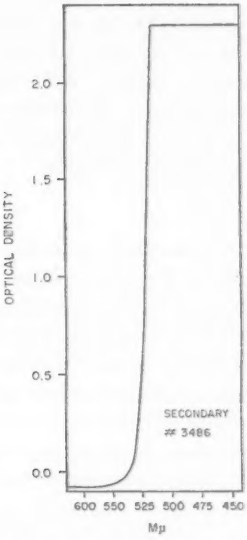



**Fig. 2.** Spectral characteristics of "yellow" secondary filter.



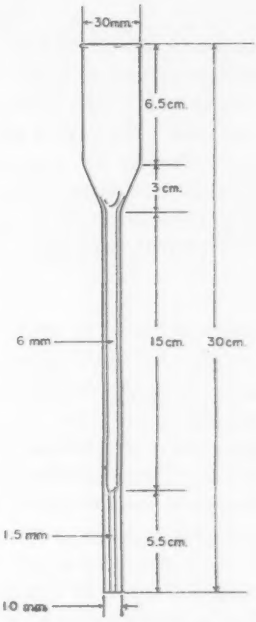




**Fig. 3.** Spectral characteristics of "blue-green" secondary filter. **Fig. 4.** A typical chromatographic column.

## STANDARDS

*Stock epinephrine:* Prepared from L-epinephrine bitartrate (Winthrop-Stearns Company), 50 μg. (as free base) per ml. in 0.1N HCl stored indefinitely in refrigerator.

Working standard: Prepared fresh daily from stock solution to give 0.05 μg. free base per ml. in 0.1N acetic acid.

*Stock norepinephrine:* Prepared from DL-norepinephrine hydrochloride (Winthrop-Stearns) 50 μg. (as free base) per ml. in 0.1N HCl, stored indefinitely in refrigerator.

Working standard: Prepared fresh daily from stock solution to give 0.05 μg. free base per ml. in 0.1N acetic acid.

## CHROMATOGRAPHIC COLUMN

One piece, consisting of reservoir of approximately 35 ml. capacity attached to a column 15 cm. long with inside diameter 6 mm. and a capillary 5 cm. long with inside diameter 1.5 mm. (Fig. 4).

## GLASSWARE

All glassware except the cuvets is washed by rinsing with tap water, soaking in a good grade of laboratory detergent, rinsing with distilled water and finally with glass-distilled water, and allowed to air-dry, inverted. The cuvets are boiled in 1:1 mixture of conc. $HNO_3$ and water, followed by rinsing as above. Cuvets are calibrated using a very dilute solution of sodium fluorescein. Starting with approximately 150 cuvets, it is possible to obtain a sufficient number which give identical readings with the fluorescein solution.

## PROCEDURE

The alumina column for extraction of the plasma is prepared as follows: A small glass-wool plug is placed in the constriction of the column, and a stopper, consisting of a piece of rubber tubing with a pinch clamp, is placed on lower end. Sufficient buffer is added to fill the narrow portion of the column, and a slurry of alumina in 2.5 ml. acetate buffer (1) is added with stirring. As the alumina settles, a small glass rod is worked gently up and down in the column to remove any air bubbles and to prevent the alumina from clumping. The stopper is then removed and when the descending buffer level has almost reached the top of the alumina column, the plasma sample is added. The sample is allowed to flow through the column. The extraction on alumina and elution of the catechol amines is carried out as described by Weil-Malherbe and Bone (1). The acetic acid eluate is transferred to a ground-glass stoppered test tube and the fluorescence developed (1). However, the fluorescent derivatives are allowed to cool at room temperature for 25 minutes. An aliquot of the isobutanol extract is obtained (1) and measured in the fluorometer. Standard solutions of 0.1 $\mu$g. of epinephrine and norepinephrine, each in 10 ml. of 0.1N acetic acid, and a reagent blank are treated in a manner similar to the eluates. Standards of 0.2 $\mu$g. can be used if the anticipated values are high as, for example, in specimens from a severely traumatized animal.

It is important that the standards be carried along with the unknown samples in every step of the analysis and that they be treated identically with respect to heating and cooling, etc., because the fluorescence of the norepinephrine derivative in aqueous solution changes with time, even after heating for 20 minutes. However, once the material has been extracted into isobutanol the fluorescence is stable for several hours.

Readings on the fluorometer are taken with each set of secondary filters. With the No. 3486 as secondary filter, the sensitivity is set to give full scale deflection with the epinephrine standard; the norepinephine standard, samples, and blank are read at this setting.

### CALCULATIONS

The concentrations of epinephrine and norepinephrine are calculated by the equations derived by Weil-Malherbe and Bone (2).

$$N = \frac{mn}{m\text{-}n}\,(b\text{-}y) \div \text{vol. of plasma}$$

$$A = \frac{n}{y\text{-}m} \div \text{vol. of plasma}$$

N = Noradrenaline concentration

A = Adrenaline concentration

$$b = \frac{\text{Reading of unknown with secondary filter nos. 5433-3384}}{\text{Reading of adrenaline standard with filter nos. 5433-3384}} \times \text{Conc. of Adrenaline standard}$$

$$y = \frac{\text{Reading of unknown with secondary filter No. 3486}}{\text{Reading of adrenaline standard with filter No. 3486}} \times \text{Concentration of adrenaline standard}$$

m = ratio of the fluorescence of epinephrine standard to fluorescence of norepinephrine standard with secondary filter no. 3486.

n = ratio of fluorescence of epinephrine standard to fluorescence of norepinephrine standard with secondary filters no. 5433-3384.

All readings corrected for blanks. In 37 determinations, carried out on different days, the following values were obtained (mean ± one standard deviation):

$$m = 1.86 \pm 0.211$$
$$n = 0.55 \pm 0.065$$

It was noted that the variation of the ratios is dependent to some extent on the method of cooling. The manner of cooling reported above gave the most reproducible results.

## RESULTS

### REPRODUCIBILITY AND RECOVERIES

The precision of the method was checked by analyzing aliquots of pooled plasma.

The results with both human and rat plasma are shown in Table 1. Percentage recoveries were determined by adding known amounts of either or both catechol amines to human and rat plasma (Table 2).

When samples contained less than 0.01 μg. in the eluate, the recoveries dropped to about 75 per cent and were variable, due largely to

**Table 1.** PRECISION OF MEASUREMENT OF EPINEPHRINE AND NOREPINEPHRINE

| Rat Plasma | | | Human Plasma | | |
|---|---|---|---|---|---|
| | Micrograms/Liter | | | Micrograms/Liter | |
| Aliquot | Epinephrine | Norepinephrine | Aliquot | Epinephrine | Norepinephrine |
| 1 | 7.6 | 26.0 | 1 | 0.3 | 3.5 |
| 2 | 7.2 | 18.9 | 2 | 0.4 | 3.3 |
| 3 | 6.2 | 21.5 | 3 | 0.4 | 3.6 |
| 4 | 8.6 | 24.4 | 4 | 0.4 | 3.1 |
| 5 | 7.4 | 20.3 | 5 | 0.7 | 3.7 |
| 6 | 7.0 | 19.6 | 6 | 0.2 | 3.1 |
| | | | 7 | 0.5 | 3.6 |
| Mean | 7.3 | 21.8 | Mean | 0.4 | 3.4 |
| Range | 6.2-8.6 | 18.9-26.0 | Range | 0.2-0.7 | 3.1-3.6 |
| Standard Deviation | ±0.79 | ±2.90 | Standard Deviation | ±0.18 | ±0.21 |

**Table 2.** RECOVERY OF EPINEPHRINE AND NOREPINEPHRINE ADDED TO PLASMA

| Rat Plasma | | | Human Plasma | | |
|---|---|---|---|---|---|
| | Epinephrine | Norepinephrine | | Epinephrine | Norepinephrine |
| No. of Expts. | 11 | 11 | No. of Expts. | 20 | 21 |
| Amounts Added | 0.02-0.2 ug | 0.02-0.2 ug | Amounts Added | 0.01-0.1 ug | 0.01-0.1 ug |
| Mean Recovery | 99% | 88% | Mean Recovery | 98% | 96% |
| Standard Deviation | ±15% | ±17% | Standard Deviation | ±17% | ±22% |

variations in the cuvets and fluctuations in the galvanometer readings at these low concentrations.

## SPECIFICITY

Weil-Malherbe and Bone (6) have reported that the substances estimated in human plasma by their method are epinephrine and norepinephine. However, their technic for the identification involved a preliminary extraction which was not used in their regular procedure. Consequently, we have examined the material which comes directly from the column. Table 3 illustrates that known epinephrine and norepinephrine come off the column unchanged. The chromatography of the catechol amines prior to being put on the column and those that have been run through the column were carried out as reported elsewhere (6).

In order to obtain a sufficient quantity of the physiologically occurring hormones, the substances were extracted from plasma of rats subjected to tumbling trauma, a condition known to elevate the level of both compounds (3). They have been identified as epinephrine and norepinephrine by chromatographic analysis of the fluorescent deriv-

**Table 3.** EFFECT OF CHROMATOGRAPHY ON EPINEPHRINE AND NOREPINEPHRINE

| Solvent System | Experiment 1 | | Experiment 2 | |
|---|---|---|---|---|
| | RF Values | | | |
| | Known | | Known (thru column) | |
| | Epi | Nor | Epi | Nor |
| Phenol-H2O (3-1) | 0.54 | 0.27 | 0.52 | 0.24 |
| | 0.54 | 0.27 | 0.59 | 0.32 |

atives. The following procedure was used: One hundred male Sprague-Dawley rats, weighing 225-275 Gms., were tumbled 600 turns in a Noble-Collip drum (7), sacrificed by decapitation and the blood collected in a solution of sodium fluoride—sodium thiosulfate as previously described (3). The pooled plasma (approximately 70 ml.) was divided into 10 ml. aliquots and each aliquot carried through the extraction and condensation with ethylene diamine in the usual manner. In order to concentrate the fluorescent derivatives for paper chromatography, the first sample was extracted with 6 ml. of isobutanol, the isobutanol extract removed and used to extract each of the remaining samples in succession.

On each of three Whatman No. 1 filter paper strips measuring 48 $\times$ 8 cm. (6) 1 ml. of the isobutanol extract and 1 ml. of an isobutanol extract containing a mixture of the fluorescent derivatives obtained from 0.1 $\mu$g. of epinephrine and 0.1 $\mu$g. of norepinephrine was spotted. The spots were applied with a pipet 2.5 cm. apart on a line 3 cm. from the bottom of the paper, and dried with a stream of warm air.

Chromatograms were developed in three different solvent systems using the ascending technic. Each chromatogram was allowed to run 20 hours. The paper was then air dried and scanned in a dark room with a hand ultraviolet lamp. Under ultraviolet light the epinephrine derivative appears as a yellow fluorescent spot and the norepinephrine derivative as a blue-green spot.

Table 4 shows the results of two separate experiments, each performed on two different groups of rats.

## RESULTS IN NORMAL SUBJECTS

In order to establish a baseline of control values for future studies on humans, plasma catechol amines were determined in normal males and females of various ages. All subjects were engaged in their regular duties. Samples were obtained at approximately 9.00 A.M. and were worked on immediately. The results are presented in Table 5. When calculated values of epinephrine were less than zero, Manger

**Table 4.** IDENTIFICATION AND CHARACTERIZATION OF EPINEPHRINE AND NOREPINEPHRINE AFTER ELUTION FROM COLUMN

| Solvent System | Experiment 1 RF Values | | | | Experiment 2 RF Values | | | |
|---|---|---|---|---|---|---|---|---|
| | Known | | Unknown | | Known | | Unknown | |
| | EPI | NOR | EPI | NOR | EPI | NOR | EPI | NOR |
| 0.2N $NH_4OH$ Sat. % Isobutanol | 0.40 | 0.27 | 0.40 | 0.27 | 0.33 | 0.24 | 0.37 | 0.21 |
| pH10 Buffer* Sat. % Isobutanol | 0.39 | 0.23 | 0.39 | 0.23 | 0.41 | 0.28 | 0.41 | 0.28 |
| Methanol - Conc. $NH_4OH$ (9:1) | 0.53 | 0.77 | 0.49 | 0.77 | 0.56 | 0.32 | 0.52 | 0.83 |

*Clark & Lubs Borate Buffer.

**Table 5.** CONCENTRATION OF EPINEPHRINE AND NOREPINEPHRINE IN NORMAL HUMAN PLASMA

| Males | | | Females | | |
|---|---|---|---|---|---|
| | Micrograms/Liter | | | Micrograms/Liter | |
| Age | Epinephrine | Norepinephrine | Age | Epinephrine | Norepinephrine |
| 19 | < 0.1 | 1.1 | 21 | 0.7 | 7.4 |
| 20 | 0.4 | 1.7 | 23 | 0.5 | 1.8 |
| 21 | < 0.1 | 1.7 | 24 | 0.6 | 4.6 |
| 22 | < 0.1 | 5.0 | 26 | < 0.1 | 4.1 |
| 23 | 0.2 | 1.9 | 31 | 0.4 | 5.5 |
| 24 | < 0.1 | 4.3 | 32 | 0.4 | 3.4 |
| 25 | < 0.1 | 1.5 | 34 | 0.5 | 5.1 |
| 25 | < 0.1 | 5.5 | 34 | 0.4 | 5.9 |
| 27 | 0.2 | 2.1 | 35 | 0.5 | 6.5 |
| 29 | < 0.1 | 3.8 | 36 | 0.6 | 5.7 |
| 30 | < 0.1 | 3.9 | 38 | 0.4 | 4.6 |
| 31 | < 0.1 | 4.8 | 45 | 0.7 | 4.5 |
| 32 | < 0.1 | 4.1 | | | |
| 35 | < 0.1 | 4.6 | | | |
| 36 | < 0.1 | 4.2 | | | |
| 40 | < 0.1 | 5.5 | | | |
| 42 | < 0.1 | 6.4 | | | |
| 43 | 0.2 | 5.4 | | | |
| 45 | 1.2 | 1.8 | | | |
| 50 | < 0.1 | 3.4 | | | |
| 51 | < 0.1 | 1.5 | | | |
| 52 | < 0.1 | 4.9 | | | |
| 53 | 0.7 | 0.6 | | | |
| 53 | < 0.1 | 1.2 | | | |
| 53 | < 0.1 | 3.7 | | | |
| 54 | < 0.1 | 2.8 | | | |
| 55 | < 0.1 | 2.9 | | | |
| 57 | 0.2 | 3.9 | | | |
| No. of samples | 28 | | 12 | | |
| Range | 0.1-1.2 | 0.6-6.4 | | 0.1-0.7 | 1.8-7.4 |
| * Mean | 0.11 | 3.4 | | 0.47 | 4.9 |
| ± S.D. of mean | ±0.05 | ±0.30 | | ±0.05 | ±0.42 |

* Values <0.1 considered as 0 for calculation of means.

*et al.* (5) reported them as zero. As stated by Manger, these negative values are the result of errors intrinsic in the method when very low concentrations are involved. In this study, such values were reported as less than 0.1 μg. per liter. The reproducibility of the concentration of the catechol amines in human volunteers from week to week is shown in Table 6. These samples were taken at the same time on the same day of each week.

**Table 6.** REPRODUCIBILITY OF THE MEASUREMENT OF EPINEPHRINE AND NOREPINEPHRINE

| Study No. | 1 | | 2 | | 3 | |
|---|---|---|---|---|---|---|
| Week | Epi | Nor | Epi | Nor | Epi | Nor |
| 1 | 0.2 | 3.4 | 0.2 | 3.5 | < 0.1 | 2.5 |
| 2 | < 0.1 | 5.7 | < 0.1 | 5.1 | < 0.1 | 4.8 |
| 3 | < 0.1 | 5.2 | < 0.1 | 6.6 | < 0.1 | 4.6 |
| 4 | < 0.1 | 5.6 | 0.2 | 6.0 | < 0.1 | 4.3 |
| 5 | < 0.1 | 5.2 | 0.2 | 4.8 | < 0.1 | 4.8 |
| 6 | < 0.1 | 2.7 | 0.2 | 4.8 | 0.2 | 6.5 |
| 7 | 0.2 | 4.7 | < 0.1 | 3.5 | 0.2 | 3.0 |
| 8 | 0.2 | 3.4 | | | 0.2 | 4.8 |
| 9 | | | | | 0.2 | 3.0 |

**Table 7.** CHANGE IN CONCENTRATION OF EPINEPHRINE AND NOREPINEPHRINE IN HUMAN PLASMA WITH AGE

| Age | Sex | Micrograms/Liters Epinephrine | Norepinephrine |
|---|---|---|---|
| Newborn | M | 0.1 | 6.8 |
| Newborn | M | 0.1 | 5.4 |
| Newborn | F | 0.1 | 4.1 |
| 8 Months | M | 0.1 | 4.4 |
| 12 Months | F | 0.5 | 2.2 |
| 12 Months | M | 0.4 | 4.3 |
| 19 Months | F | 0.1 | 3.2 |
| 71 Years | M | 0.8 | 6.0 |
| 78 | M | 0.5 | 5.9 |
| 81 | M | 0.5 | 5.8 |
| 81 | M | 0.3 | 6.8 |
| 82 | M | 1.4 | 8.0 |
| 86 | M | 0.6 | 6.1 |

It should be noted that the epinephrine values are considerably lower than those reported by Weil-Malherbe and Bone (2). The latter reported average values of 1.18 and 1.46 $\mu$g. per liter of plasma in 22 males and 21 females, respectively. In our study, if all negative values are taken as zero, the calculated means are 0.11 and 0.47 $\mu$g. per liter of plasma in males and females, respectively. The consistently higher concentration of epinephrine ($P<0.001$) and norepinephrine ($P<0.01$) in females must be noted.

Included in this study were determinations on subjects in the extreme age groups, i.e., infants and elderly men (Table 7). Cord blood was obtained at time of birth and samples were obtained from babies brought in for routine examinations. The elderly men were volunteers from among the occupants of the Old Soldiers' Home in Washington, D. C. In general, both epinephrine and norepinephrine concentrations in the elderly subjects were higher than in the younger group of males. However, to demonstrate a significant difference in the elderly subjects, more determinations would be necessary.

## SUMMARY

The Weil-Malherbe and Bone method for the analysis of the plasma concentration of epinephrine and norepinephrine has been modified and used to determine the plasma concentration in normal humans.

The average value for epinephrine in the plasma of men in this series was less than 0.11 μg./L.; for women it was 0.47 μg./L. The average value for norepinephrine in men was 3.4 μg./L. and in women it was 4.9 μg./L. The differences between the concentrations in men and women are significant, $P<0.001$ and $P<0.01$ for epinephrine and norepinephrine, respectively.

Evidence has been presented to indicate the specificity of the method. Reproducibility and recovery data have been given.

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# Protein Level in Cerebrospinal Fluid

## An Evaluation of Some Methods of Determination

*Shirley Sethna and M. U. Tsao*

THIS STUDY WAS UNDERTAKEN IN AN ATTEMPT to evaluate some of the more promising methods for the determination of cerebrospinal fluid protein with the objective of deciding on the most suitable one for use in this laboratory. There are a number of methods available, with great variations in the ease of performance and the accuracy of results; therefore it seems that some sort of compromise must be made to find a practicable method. We chose the methods for investigation on the basis of two considerations: first, their reported good correlation with results obtained by the Kjeldahl procedure, this method being the one generally accepted for its reliability; and second, on sample size required which is a necessary criterion for us, since this laboratory is routinely given a sample of no more than 1 ml. for this determination.

A comparative study was made on a series of pooled cerebrospinal fluids, and also on a series of serum and plasma specimens that had been diluted with saline to give protein concentrations in the spinal fluid range. The following methods were used simultaneously: micro-Kjeldahl; the tyrosine equivalence method of Johnston and Gibson; the sulfosalicylic acid method; the trichloroacetic acid method run at 37°; and two biuret procedures.

The tyrosine equivalence method of Johnston and Gibson (1) has had very favorable reports from nearly every source, the main objec-

From the Department of Pediatrics and Communicable Diseases, University of Michigan Medical School, Ann Arbor, Mich.

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tions to it being the procedural difficulty and time required as compared to some other methods and the interference caused by cerebrospinal fluid preservatives used in some laboratories (e.g., merthiolate (2), thymol). The sulfosalicylic acid method (3) is in general use in many laboratories, the main objection to it being that the results are not reliable in samples with abnormal albumin:globulin (A/G) ratios. The trichloracetic acid precipitation method (2) is reported to give the same value for the same total protein at all A/G ratios, and the main objection to it, the change in the results with changes in temperature, has purportedly been overcome by allowing the samples to stand in a 37° water bath for 10 minutes before reading (4). The biuret method has been shown to give excellent results for serum and has been adapted to the determination of cerebrospinal fluid protein.

## EXPERIMENTAL

All the above methods were used simultaneously on a series of diluted sera and spinal fluids. Some of the sera (in some cases plasma was used) were fresh and some had been frozen. They were diluted with physiologic saline to give protein concentrations in the normal cerebrospinal fluid range. The cerebrospinal fluids were pooled specimens, except in a few cases where the protein was so abnormally high that a large enough specimen could be obtained by diluting the small amount available. The specimens were frozen as soon as possible after they reached the laboratory and when enough had accumulated they were thawed, pooled, and centrifuged before use. If necessary they were diluted with physiological saline to give protein values in the normal spinal fluid range. It is unfortunate that it was necessary to use pooled specimens because this automatically eliminated the possibility of a very abnormal A/G ratio, which is one of the main aspects of this problem which needs to be investigated. However, there are a few sera included in the study which have abnormal A/G ratios and it seems to be generally accepted that the same proportions of constituent protein fractions prevail in cerebrospinal fluid protein as in that of the serum (5).

The "distillation" step in the micro-Kjeldahl nitrogen determination was done using Conway diffusion cells, in an adaptation of the method described by Conway (6). One milliliter of cerebrospinal fluid or its equivalent in diluted serum or plasma was used for each determination. Since the NPN is so large in relation to the total protein nitrogen in cerebrospinal fluid (7), it has been shown (3) that much

better results are obtained by determining the precipitated protein than by attempting to determine it by using the difference between the total nitrogen and NPN. This method was therefore used. The procedure for the precipitation of proteins is the same as that described below for the tyrosine equivalence method.

The standard curves for all the other methods were made simultaneously using dilutions of a fresh pooled serum that had been determined by the above method and also by a micro-Kjeldahl method with distillation in an all-glass apparatus (8). Two pooled sera were used, and the average values taken. Ten different dilutions were made of each serum, the values ranging from 6 to 115 mg. per 100 ml. Smooth, reproducible curves were obtained with all the methods, except that the sulfosalicylic method appeared to lose its reproducibility above 60 mg. per 100 ml. and the trichloroacetic method above 80 mg. per 100 ml. Therefore specimens with protein levels above those values were diluted before analysis by these procedures.

The Klett-Summerson colorimeter was used for the biuret, sulfosalicylic, and trichloroacetic acid methods, and the Evelyn colorimeter was used for the tyrosine equivalence method.

All the determinations were done in duplicate, except the micro-Kjeldahl which was run in triplicate.

All the methods were used as described in the references given, with only minor adaptations being made to allow the use of a 1 ml. sample size, except for the tyrosine equivalence method which was run as follows:

To 1 ml. of cerebrospinal fluid, add 1 ml. of distilled water and 1 ml. of 10% tricholoroacetic acid in a 12-ml. centrifuge tube. Mix by tapping the tube, allow to stand 30 minutes and centifuge until the supernatant is absolutely clear and the precipitate is well packed. Remove the supernatant by aspiration, using a long capillary pipet. To the precipitate add 0.4 ml. of 2.5N NaOH, and allow the precipitate to dissolve completely by rotating the tube so that the hydroxide contacts the sides up to about an inch. Cover the top of the tube with aluminum foil and place in a boiling water bath for 10 minutes. Cool, transfer quantitatively to an Evelyn tube, rinsing with water until the total volume of liquid in the Evelyn tube is 4.7 ml. Add 0.3 ml. of phenol reagent with mixing during the addition, add 5 ml. of distilled water, allow to stand 10 minutes and read at 520 m$\mu$ on the Evelyn colorimeter against a blank containing 0.4 ml. of 2.5N NaOH and 0.3 ml. of phenol reagent in 9.3 ml. of distilled water. The calculation is

made by determining the tyrosine equivalent from a curve previously prepared from a standard tyrosine solution, and multiplying by a factor determined on a serum specimen of known protein as determined by the micro-Kjeldahl method. The factor we found was as follows: mg. per 100 ml. tyrosine $\times$ 12.7 = mg. per 100 ml. protein.

## RESULTS

The results of the cerebrospinal fluid study are presented in Table 1 and of the serum and plasma study in Table 2. The adaptation of the tyrosine equivalence method which was used gave consistently good results on both serum and cerebrospinal fluid at all concentrations and all A/G ratios.

The sulfosalicylic and trichloroacetic acid methods gave quite good results on the pooled cerebrospinal fluids, and also on serum as long as the A/G ratios were normal. However, in three serum specimens with A/G ratios below 1, the sulfosalicylic acid method gave consider-

**Table 1.** Comparison of Cerebrospinal Fluid Protein Values by Different Methods

| *Micro-Kjeldahl* | *Tyrosine equivalence* | *Sulfosalicylic acid* | *37° trichloroacetic acid* | *Weichselbaum biuret* | *Andersch biuret* |
|---|---|---|---|---|---|
| 36.0 | 35.3 | – | – | 49.2 | – |
| 100.0 | 101.7 | – | – | 107 | – |
| 82.0 | 84.5 | – | – | 102.8 | – |
| 54.7 | 55.3 | 55 | – | 72.0 | – |
| 68.4 | 68.5 | – | – | 80.3 | – |
| 66.4 | 65.2 | – | – | 76.3 | – |
| 56.7 | 54 | – | – | 63.0 | – |
| 58.3 | 56.7 | 60.0 | – | 71.0 | – |
| 35.7 | 35.7 | 38.4 | 35.8 | 47.5 | 44.5 |
| 28.5 | 29.1 | 32.6 | 30.6 | – | 33.8 |
| 25.8 | 25.9 | 28.2 | 26.5 | – | 30.7 |
| 38.7 | 36.0 | 45.0 | 40.2 | – | 44.4 |
| 48.7 | – | 46.8 | 49.5 | – | – |
| 20.4 | – | 19.9 | 19.3 | – | 23.4 |
| 52.3 | 48.9 | 52 | 50.6 | – | 56.0 |
| 58.9 | 57.4 | – | 61 | – | 66.6 |
| 34.2 | 34.4 | 31.8 | 31.2 | – | 37.0 |
| % Kjeldahl | | | | | |
| Range | 93–103 | 93–114 | 91.5–107 | 107–137 | 107–125 |
| Avg. | 98.8 | 103.6 | 100.3 | 122.2 | 115.1 |
| Diff. from Kjeldahl | | | | | |
| Range | −3.4–2.5 | −2.4–6.3 | −3.0–2.1 | 6.3–20.8 | 2.8–8.8 |
| Avg. | −0.5 | 1.2 | 0.2 | 13.1 | 5.2 |

Table 2. Comparison of Diluted Serum and Plasma Values by Different Methods

| *Micro-Kjeldahl* | *Tyrosine equivalence* | *Sulfosalicylic acid* | *37° trichloroacetic acid* | *Weichselbaum biuret* | *Andersch biuret* | *A/G* |
|---|---|---|---|---|---|---|
| 19.1 | 19.5 | 16 | 23 | 19.4 | 21.3 | 0.43 |
| 26.7 | 25.6 | 32.1 | 28.7 | 25.9 | 26.7 | – |
| 29.3 | 27.8 | 31.3 | 29.5 | 31.0 | 30.3 | – |
| 31.4 | 31.4 | 29.2 | 33.8 | 30.7 | 35.3 | 1.2 |
| 31.6 | 32.3 | 25.3 | 43 | 31.9 | 38.3 | 0.43 |
| 33.9 | 32.8 | 37 | 33.6 | 33.8 | 35.3 | 1.3 |
| 45.7 | 45.8 | 46 | 47 | 45.0 | 48.4 | – |
| 47.1 | 49.8 | 46.2 | 46.9 | 49.8 | 51.6 | – |
| 53.7 | 52.2 | 53.2 | 51.3 | 55.1 | 57.1 | – |
| 58.0 | 59.1 | 57.8 | 57.3 | 58.1 | 65 | – |
| 28.3 | 27.3 | 26.2 | 25.9 | .. | 28.5 | – |
| 53.8 | 53.1 | 49.8 | 53.9 | .. | 55.9 | – |
| 81.3 | 80.2 | .. | | .. | 84 | – |
| 63.4 | 65.9 | .. | .. | .. | 66.7 | – |
| 30.8 | 32.8 | 26.1 | 38 | .. | 29 | 0.98 |
| 49.9 | 52.2 | 51 | 52.1 | .. | 53.5 | 1.0 |
| 85.3 | 85.4 | .. | .. | .. | 83 | 1.1 |
| 62.5 | 64.9 | .. | 62.8 | .. | 65 | – |
| 63.0 | 64.9 | .. | 65 | .. | 66.6 | .. |
| 47.1 | 46.9 | 42.5 | 46 | .. | 48 | .. |
| 40.9 | 38.3 | 43 | 42 | .. | 41.5 | .. |
| 50.0 | 52.3 | 47.5 | 47.3 | .. | 50.5 | 0.89 |
| % Kjeldahl | | | | | | |
| Range | 93.5–106.5 | 80–120 | 91.5–136 | 97–106 | 94–112 | |
| Avg. | 100.7 | 97.2 | 104.6 | 101.1 | 105.1 | |
| Diff. from Kjeldahl | | | | | | |
| Range | −2.6–2.7 | −6.3–5.4 | −2.4–11.4 | −0.8–2.7 | −2.3–7.0 | |
| Avg. | 0.4 | −1.6 | 1.3 | 0.4 | 2.2 | |

ably lower results than the micro-Kjeldahl procedure while the trichloroacetic acid method gave higher results.

Both the biuret procedure used gave excellent results on serum at all concentrations. However, when cerebrospinal fluid was used they both gave results consistently higher than those obtained with the micro-Kjeldahl method.

## DISCUSSION

The adaptation of the tyrosine equivalence method which was used gave consistently good results on both serum and cerebrospinal fluid at all concentrations and A/G ratios. There is inherent in the method a small error when the A/G ratio is abnormal, since the color due to

a given amount of globulin is slightly more intense than that which would be obtained from the same amount of albumin, and the factor for total protein is based on the average normal concentrations of these two constituents, but this error is not of great enough magnitude to change the results appreciably. Once the tyrosine equivalence factor is determined for the particular set of conditions used, it is not necessary to recheck new reagents against a standard serum, since the curves are made using a standard tyrosine solution. This method is more time consuming than the others (except micro-Kjeldahl) but it does seem to be by far the best from the standpoint of reliability of results.

It might be of interest to report here some results that we obtained using the tyrosine equivalence method without precipitating the protein. (A different factor must of course be used for the calculation.) A series of plasmas of different protein concentrations were checked against the micro-Kjeldahl method (9), and also a number of determinations were made on the same plasma at various dilutions. It appears that in samples of high protein level the values are lower than values by the micro-Kjeldahl method; in samples of low protein level the results are higher. Also in a series of different dilutions of the same serum, the greater the dilution, the higher the result that is obtained. However, in a normal range the results are good and differences are not too great unless the specimen protein content deviates considerably from normal. This method (without precipitation) was also tried for cerebrospinal fluid protein, and the results were all too high, ranging from 120 to 180 per cent of the micro-Kjeldahl value in specimens that did not require dilution, the specimens with the least protein being the farthest from the micro-Kjeldahl value. In specimens of such high protein level that large dilutions were necessary, the result approached the micro-Kjeldahl value. (One spinal fluid with a protein of 1725 mg. per 100 ml. when diluted 1:25 gave only 102 per cent of the micro-Kjeldahl value.)

It appears that there may be some substance in fairly constant amount normally appearing in cerebrospinal fluid which does not precipitate with the protein and which causes interference in this method.

The error in the total protein values obtained with the sulfosalicylic acid method on cerebrospinal fluids with abnormal A/G ratios has been reported before (2), but the same investigators found the tri-

chloroacetic method to be unaffected by changes in the A/G ratio in a study using artificial mixtures which had been made by precipitating the globulin to separate components. It is not necessarily the abnormal A/G ratio, but perhaps some interfering substances occasionally appearing in the same conditions affecting the abnormal A/G ratios, that also caused the high trichloroacetic acid value with low A/G ratios in our study. Some of the specimens with ratios below or just slightly above 1 did not show this property. In any case, it seems that this deserves further investigation.

Two different biuret methods were tried. The first one (10) was chosen because the reagent (11) had previously been used in this laboratory and had been found to give excellent results on serum and plasma at all concentrations and A/G ratios. However, when it was used for spinal fluid it was found that the results were consistently higher than the micro-Kjeldahl results. It was felt that perhaps some substance present in cerebrospinal fluid but not in serum might be giving the biuret color reaction or enhancing the color. To make sure that this was not merely true of this one biuret reagent, another biuret method (12) was tried and it also gave higher values than the micro-Kjeldahl method on cerebrospinal fluids. (Correction was made for plasma blanks.) In both cases if the biuret results are plotted against the micro-Kjeldahl results a fairly smooth curve is obtained, which makes it appear that if the standard curves were made up using cerebrospinal fluid instead of serum the results would be greatly improved. There is one other disadvantage of the biuret method however. Since the protein concentration of the cerebrospinal fluid is so low in normal specimens, even though the specimen is used undiluted, the color obtained is not intense enough to give the same reproducibility as the other methods. Also in the Andersch method (12) the color is not very stable and tends to rise on standing in some cases, as described by Andersch.

After this study was completed, a new method was brought to our attention (13). This method is based on the amount of a dye used up in precipitating the protein in a sample of cerebrospinal fluid. It has some disadvantages: (a) the samples must be read on the Beckman spectophotometer; (b) standard curves must be set up on pure albumin and globulin; and (c) both albumin and globulin must be determined on any specimen containing a mixture of the two. The amount of sample required is small though, and could probably be further reduced, and this appears to be a relatively simple method

for determining the A/G ratio of cerebrospinal fluid in cases where it is of interest.

## SUMMARY

Results are given of simultaneous determinations of protein on 17 pooled cerebrospinal fluids (see Table 1) and 22 diluted sera and plasma specimens (see Table 2) by the following methods: micro-Kjeldahl; Johnston-Gibson tyrosine equivalence method; sulfosalicylic acid method; trichloroacetic acid method at 37°; and two biuret methods. The micro-Kjeldahl method was used as the standard for comparison.

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# Urinary Sulfur Partition in Normal Men and in Cancer Patients

*Daphne B. Papadopoulou*

INFORMATION RELATING TO URINARY SULFUR partition of normal men and of cancer patients has been limited. Folin (1), Clark (2), Pirie (3), and more recently Freyberg (4), Mendes (5), Du Vigneaud (6), and some other investigators (7, 8) have explored the over-all sulfur economy and urinary excretion in normal adults. Müller (8) has reported the amount of excretion of neutral sulfur in 1 patient with cancer of the liver.

Crabtree (9) reported variations of neutral sulfur excretion in urine in mice after treating them with carcinogens.

Our experiments deal with the partition of inorganic sulfate, total sulfate, total sulfur, and neutral sulfur in urine of 10 normal men and 27 cancer patients.

## EXPERIMENTAL

The total and inorganic sulfate was determined by the method of Fiske (10) in 200-ml. aliquots of 24-hr. specimens of urine. The phosphate was removed by addition of 20 ml. of 12.5% ammonium chloride, 3 Gm. of magnesium carbonate, and sufficient ammonium hydroxide to make the solution alkaline to phenolphthalein in a final volume of 200 ml., after Turner *et al.* (11).

Inorganic sulfate and total sulfate determinations were made in duplicate on 50-ml. aliquots of the filtrate, the first before and the last after hydrolysis with 1 ml. of 3 N hydrochloric acid. The total

From the Greek Cancer Institute, Athens, Greece.

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Present address: National Cancer Institute, Bethesda, Md.

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sulfur was determined in duplicate on 25-ml. aliquots of the urine filtrate after digesting with 15 ml. of Pirie's reagent (3) in a Kjeldahl flask for 3 to 4 hours. The remaining perchloric acid was boiled off over a free flame.

The black residue was dissolved in a small quantity of 6N HCl, redigested, dissolved in water, and the sulfur determination was made by titration of the benzidine sulfate precipitate with .02N NaOH, using phenol red as the indicator.

The value of neutral sulfur was obtained by subtracting the total sulfate from the total sulfur.

The total nitrogen was also estimated according to the method of Kjeldahl.

## CLINICAL MATERIAL

Normal subjects consisted of healthy males and females, middle aged, assembled from the clinic personnel.

The cancer patients had cancer of the lung, breast, larynx, gastrointestinal tract, and stomach, with positive histologic findings in all cases.

Some of these also showed clear evidence of metastasis but not any evidence of cachexia. Both normal subjects and patients were on normal diet.

## RESULTS AND DISCUSSION

The data are shown in Tables 1, 2, and 3. Table 1 shows the values of inorganic and total sulfate, total and neutral sulfur, and nitrogen

**Table 1.** URINARY SULFUR PARTITION OF NORMAL SUBJECTS (MEQ.[a] SULFUR OF 24-HOUR URINE)

| *Case no.* | *Inorganic sulfate* | *Total sulfate* | *Total sulfur* | *Neutral sulfur* | *Neutral/total sulfur (%)* | *Total nitrogen mg./24 hr.* |
|---|---|---|---|---|---|---|
| 1. | 28.0 | 28.0 | 34.0 | 6.0 | 17.65 | 3.2 |
| 2. | 20.0 | 24.0 | 35.0 | 11.0 | 31.43 | 6.4 |
| 3. | 56.0 | 61.1 | 72.0 | 10.9 | 15.19 | 7.0 |
| 4. | 67.1 | 77.0 | 87.0 | 10.0 | 11.49 | 8.2 |
| 5. | 41.1 | 44.0 | 51.6 | 7.6 | 14.73 | 8.4 |
| 6. | 23.0 | 26.0 | 36.0 | 10.0 | 27.78 | 14.5 |
| 7. | 32.5 | 49.4 | 52.0 | 2.6 | 5.00 | 1.9 |
| 8. | 29.2 | 35.3 | 40.0 | 4.7 | 11.75 | -- |
| 9. | 57.2 | 71.8 | 79.0 | 7.2 | 9.11 | -- |
| 10. | 25.7 | 31.5 | 40.0 | 8.5 | 21.25 | 13.4 |

[a] 2 mEq. = 1 mm. of sulfur

in the urine of 10 normal men. Table 2 shows the same determination in 27 cancer cases. Table 3 shows the mean values of inorganic, total sulfate, and total and neutral sulfur of normal men and cancer patients. Also shown are the mean values of total-nitrogen excretion as well as the ratio of total sulfur to total nitrogen. The data show that the mean value of excretion of inorganic sulfate in cancer patients is about 30 per cent lower in cancer patients than in normal.

The value of total sulfate is also lower by about 15 per cent in cancer patients. There was no change in the mean value of the total sulfur excreted or the ratio of total sulfur to total nitrogen. The most significant difference we have found was in the excretion of neutral sulfur. The mean value was found to be 17.12 mEq. in cancer patients,

**Table 2.** URINARY SULFUR PARTITION OF CANCER PATIENTS (MEQ. SULFUR OF 24-HOUR URINE)

| *Case no.* | *Inorganic sulfate* | *Total sulfate* | *Total sulfur* | *Neutral sulfur* | *Neutral/total sulfur (%)* | *Total nitrogen mg./24 hr.* |
|---|---|---|---|---|---|---|
| 1 | 21.0 | 57.0 | 113.0 | 56.0 | 49.55 | 5.7 |
| 2 | 62.0 | 86.0 | 145.5 | 58.5 | 40.20 | 7.6 |
| 3 | 15.0 | 36.0 | 76.0 | 40.0 | 52.63 | 6.0 |
| 4 | 74.0 | 95.0 | 132.0 | 37.0 | 28.03 | 9.0 |
| 5 | 17.0 | 19.0 | 25.0 | 6.0 | 24.00 | 4.1 |
| 6 | 18.0 | 25.2 | 36.0 | 10.8 | 30.00 | 11.2 |
| 7 | 29.0 | 34.0 | 58.0 | 24.0 | 41.37 | 14.3 |
| 8 | 33.0 | 45.0 | 68.0 | 23.0 | 33.82 | 4.1 |
| 9 | 5.3 | 7.2 | 61.0 | 53.8 | 88.19 | 15.4 |
| 10 | 24.5 | 25.6 | 37.5 | 11.9 | 31.73 | 6.1 |
| 11 | 15.0 | 29.9 | 35.0 | 5.1 | 14.57 | 6.9 |
| 12 | 52.7 | 59.0 | 87.0 | 28.0 | 32.18 | 9.3 |
| 13 | 27.5 | 40.1 | 46.9 | 6.8 | 14.49 | 11.1 |
| 14 | 25.2 | 32.7 | 40.0 | 7.3 | 18.25 | 4.3 |
| 15 | 13.0 | 22.0 | 26.0 | 4.0 | 15.38 | 4.9 |
| 16 | 25.0 | 94.0 | 120.0 | 26.0 | 21.66 | 15.2 |
| 17 | 14.0 | 19.0 | 22.0 | 3.0 | 13.63 | 9.8 |
| 18 | 14.4 | 22.2 | 25.8 | 3.6 | 13.95 | 7.2 |
| 19 | 16.7 | 27.3 | 33.6 | 6.3 | 18.75 | 8.6 |
| 20 | 27.5 | 43.7 | 48.3 | 4.6 | 9.53 | 8.7 |
| 21 | 22.9 | 32.6 | 43.1 | 10.5 | 24.36 | .. |
| 22 | 31.1 | 42.3 | 50.3 | 8.0 | 15.90 | 3.7 |
| 23 | 33.1 | 35.9 | 44.9 | 9.0 | 20.04 | 3.5 |
| 24 | 11.9 | 17.0 | 21.1 | 4.1 | 19.43 | 5.1 |
| 25 | 14.1 | 17.3 | 19.2 | 1.9 | 9.85 | 2.2 |
| 26 | 8.4 | 9.6 | 12.5 | 2.9 | 23.20 | 6.9 |
| 27 | 15.4 | 25.1 | 36.5 | 10.4 | 28.49 | 6.3 |

**Table 3.** Mean Value in mEq. of Sulfur Partition in Urine of Normal Men and Cancer Patients

| | *Normal* | | | *Cancer* | | | |
|---|---|---|---|---|---|---|---|
| | *No. cases* | *Mean value mEq. of sulfur* | *Per cent of the total sulfur* | *No. cases* | *Mean value mEq. of sulfur* | *Per cent of the total sulfur* | *t[a] value* |
| 1. Inorganic sulfate | 10 | 37.98 | 75.92 | 27 | 24.76 | 45.68 | 2.25[b] |
| 2. Total sulfate | 10 | 44.81 | 85.09 | 27 | 37.03 | 68.32 | 1.906 |
| 3. Total sulfur | 10 | 52.66 | 100.00 | 27 | 54.20 | 100.00 | 0.129 |
| 4. Neutral sulfur | 10 | 7.85 | 14.92 | 27 | 17.12 | 31.44 | 2.660[c] |
| 5. Total N mg. in 24 hr. | 8 | 6.94 | -- | 26 | 7.59 | -- | 0.360 |
| Ratio total sulfur /total nitrogen | -- | -- | 12.14 | -- | -- | 11.42 | |

[a] *t* test of significance of the difference between the means.

[b] Significant at 0.05 level, 35 degrees of freedom.

[c] Since it is obvious that the variances of the two distributions involved in (4) can not be assumed to be equal, the Welch procedure is indicated with the above result (21). Significant at .05 level, 24 degrees of freedom.

as compared with 7.85 mEq. in a 24-hr. specimen of urine in normal subject.

This corresponds to 31, 44 per cent of total sulfur in cancer and about 15 per cent in normal, and a ratio of 2 to 1.

The over-all economy and metabolism of sulfur is not fully understood. Different investigators (4,12,13) have also reported variations in the excretion of different sulfur fractions.

The inorganic sulfate, which constitutes about 80 per cent of the total sulfur excretion in normal men (12, 13), is derived mainly from the oxidation of organic substances and therefore represents the end products of sulfur oxidation in the body.

In studies of Laidlaw and Dziewiatkowsky (14,15) inorganic sulfate labeled with radioactive sulfur was given to normal men, 80 to 90 per cent of the radioactivity excreted was found in the inorganic sulfate fraction of the urine. A very small amount, 5 to 6 per cent was excreted as ethereal sulfate, that is, inorganic sulfate conjugated with phenolic derivatives

Müller (16), Pirie (3), and other investigators (5), have reported that in men methionine was readily oxidized to sulfate. The neutral sulfur fraction in urine (8,9,12,13,17) consists of a mixture of unchanged organic sulfur compounds such as the sulfur-containing amino acids, cystine and methionine, taurine mercapturic acid (7), and some volatile sulfides. Müller (16), Freyberg (4), Duncan (12),

and Hurxthal (13) reported also that while the inorganic sulfate is formed from the catabolism of dietary sulfur proteins, the neutral fraction of sulfur is derived mainly from endogenous body protein.

The neutral sulfur fraction which represents the intracellular oxidation of body proteins, as well as sulfur from unoxidized sulfur-containing organic substances such as taurine, which is derived from the bile salts, and sulphydryl-containing cell constitutents of unknown nature (9, 12), is a more absolute value than inorganic sulfate and appears constant in urine. It varies in different species and in men makes up about 5 to 20 per cent (4) of the total sulfur, according to some investigators (4, 8, 12, 13). Turner (11), and other (3, 16) show that a metabolic relationship exists between the excretion of neutral sulfur and methionine in mice. Although a relative increase in the neutral sulfur is observed in inanition, a protein-poor diet, and cases of low protein catabolism (8,13), our cancer patients did not show any evidence of low protein catabolism that was indicated in the excretion of total nitrogen and the ratio of total sulfur to total nitrogen (4,8), which was about the same as in normal subjects. These results support the view that there may be a decrease in the rate of the intracellular oxidation of the sulfur-containing amino acids (5,11) and other substances which are not oxidized to sulfate but are excreted unchanged in the fraction of neutral sulfur.

These findings also raise the question of whether cancer patients have a changed biochemical metabolic pattern, regarding the biochemical reaction of transulfuration and transmethylation in the body (6, 18). Whether it is merely a cachectic change of the biochemical pattern and not a characteristic of neoplastic disease, it requires further investigation.

Nevertheless, the fact that some interference with intracellular sulfur reaction (9,17,19) has been observed in mice treated with carcinogens and the SH-containing cellular constituents fixed by carcinogenic substances (20) offers some support to the view for the former explanation.

## SUMMARY

1. The partition of inorganic and total sulfate and total and neutral sulfur has been estimated in 10 normal men and 27 cancer patients.

2. The excretion of inorganic and total sulfate was found to be

decreased and the neutral sulfur fraction increased in cancer patients more than in the normal subjects.

3. From the data above, it is suggested that there may be a decrease in the rate of the intracellular oxidation substances in the body of cancer patients.

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# Partition Chromatography of Organic Acids in Body Fluids with Silica Gel

## Application of the Method to Normal Human Urine

*Samuel Meites*

In 1941, Martin and Synge, in their now classical paper, introduced silica gel as an inert stationary phase in the partition chromatography of acetylated amino acids (1). The use of silica gel was subsequently extended to the separation of straight chain saturated fatty acids (2-5). Isherwood (6) introduced silica gel as a medium for the separation of various volatile and non-volatile acids, particularly in fruit, as well as successfully using it for the extraction of the acids. Isherwood's method was subsequently extended and modified by several workers (7-13). Frohman, Orten, and Smith (9) separated acids of the citric acid cycle from rat tissues with the aid of silica gel columns. They were able to report quantitative values for lactic, citric and pyruvic acids in blood. In this paper, use of the silica gel column for the separation of non-α-amino organic acids in human urine is described.

### METHOD OF EXTRACTION

The method of Isherwood may be successfully applied to the extraction of the organic acids in urine. Preliminary unpublished results indicate that the extraction procedure is also applicable to sweat, saliva, spinal, and amniotic fluids. For urine the method routinely followed has been to collect a 24-hour specimen, the urine being stored

From the Clinical Laboratory, Children's Hospital, and the Department of Pediatrics, Ohio State University College of Medicine, Columbus, Ohio.

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in a refrigerator or freezer during the time of collection. The volume of urine is noted, and a 15 ml. portion is adjusted to *p*H 9.8 with 5N NaOH and indicator paper. It is then evaporated to dryness in vacuo at 35° in a Rinco evaporator, and then, if necessary, stored in a freezer. Two milliliters of water are then added to dissolve the residue and the solution is adjusted to pH 2.0 with 5N $H_2SO_4$ and indicator paper. Sufficient silica gel is added to absorb all of the liquid, and give the appearance of a free-flowing powder. The powder is transferred to an extraction thimble (Corning No. 33950, LC) containing one thickness of Whatman No. 1 filter paper. Four hundred milliliters of 50% butanol-chloroform (v/v), saturated with 0.5N $H_2SO_4$ are then passed through the column. The flask from which the silica gel was removed is repeatedly washed with the solvent, and each washing transferred to the column.

The extract is poured into a separatory funnel. Twenty milliliters of distilled water are added and the solution adjusted to *p*H 9.8, as before. The funnel is shaken vigorously for 2 minutes and allowed to stand 30 minutes. The supernatant is then withdrawn and saved (refrigerate), while the extraction is repeated twice using 10 ml. of water in the second and 5 ml. in the third. The *p*H is not adjusted in the last two extractions. The combined extracts are then evaporated to dryness in the Rinco evaporator and stored in a freezer until ready for analysis.

## CHROMATOGRAPHIC ANALYSIS

The standard survey column of Bulen, Varner, and Burrell (10) is used for the analysis of the non-α-amino organic acids, with minor changes in procedure. Eight-and-a-half grams of silica gel are ground with 6.0 ml. of 0.5N $H_2SO_4$ before packing the column. To the dry extract of urine is added 0.3 ml. of water and then 5N $H_2SO_4$ (usually about 4 drops) until the *p*H of solution is 2.0 to indicator paper. One gram of silica gel is added and the free flowing powder is transferred to the column as described by Bulen *et al.* A slight positive pressure is maintained on the solvent in the reservoir by use of a sphygmomanometer bulb and valve attached to a liter bottle which in turn is connected to the solvent reservoir. The butanol-chloroform solvent system of Bulen *et al.* is employed with omission of the 50 per cent mixture.

The effluent passes through an intermittent siphon arbitrarily set to deliver 3.7 ml. Individual fractions are collected in 19 x 150 mm. test

tubes in an automatic fraction collector.[1] One drop of phenol red is added to each tube and titration is performed with 0.005N NaOH, without the addition of distilled water. A total of 172 fractions is collected.

With each new batch of silica gel (Mallinckrodt's silicic acid, 100 mesh, suitable for chromatographic analysis) a blank run, with water replacing extract, must be performed in order to quantitatively ascertain the amount of sulfuric acid eluted from the column. This may be as much as 12 to 18 ml. of 0.005N NaOH during the course of collecting 172 fractions.

## RESULTS

Table 1 shows the recovery of a few acids which were subjected to the extraction procedure of Isherwood followed by silica gel chromatography. Recovery of all acids tested is quantitative with the exception of the readily decarboxylated α-keto acids.

The order of elution of 42 organic acids which may occur in body fluids is given in Table 1. The range and peak of elution of each acid may vary slightly with each batch of silica gel. Baldwin *et al.* (12) have suggested a method for standardizing silica gel to give constant threshold volumes, but this has, thus far, proved unnecessary for the analysis of urinary acids with the procedure adopted.

A comparison of the Van Slyke and Palmer titration (14, 15) of total organic acids in urine with the amount of acid obtained by the silica gel method, using the urine of 10 adult subjects, was made. The results indicate that 40 to 80 per cent of the acid titrated by the Van Slyke and Palmer method is recovered by the silica gel method. The titration values were not corrected for creatinine, creatine, and amino acids, hence the actual amount recovered by silica gel would be considerably greater. The undetermined acid represents the group shown in Table 1 which are not eluted in the 172 fractions collected.

The results given below are based on experience gained with the analyses of 80 urines.

Figure 1 illustrates the typical chromatogram obtained from human urine. To more positively establish the identity and number of acids in each peak represented, the individually titrated fraction from identical peaks of several urines were pooled and evaporated to dryness. They were than subjected to paper chromatography by several methods.

[1]Autonomos Instruments, 244 E. Royal Forest Blvd., Columbus, Ohio.

**Table 1.** Elution Range and Peak Fraction of Biologically Significant Organic Acids from Silica Gel Columns

| *Acid* | *Elution range*[a] | *Peak fractions*[a] | *Acid* | *Elution range*[a] | *Peak fractions*[a] |
|---|---|---|---|---|---|
| Acetyl salicylic | 3–10 | 3–5 | Malonic | 48–65 | 51 |
| Benzoic | | | α-Ketoglutaric | 45–60 | 53 |
| Butyric | | | Pyrrolidone carboxylic | 50–75 | 54 |
| Cholic | | | trans-Aconitic | | |
| Hippuric | | | Oxalacetic | 55–75 | 67 |
| m-Hydroxybenzoic | | | p-Aminobenzoic | 64–84 | 69–70 |
| p-Hydroxybenzoic | | | Oxalic | 71–90 | 76 |
| p-Hydroxyphenyllactic | | | Tricarballylic | 70–85 | 78 |
| p-Hydroxyphenylpyruvic | | | cis-Aconitic | 75–90 | 84 |
| Indole-3-acetic | | | Glycolic | | |
| Phenaceturic | | | Malic | 105–125 | 115 |
| Phenylpyruvic | | | Glyoxylic | 120–trails | 130 |
| Phenyllactic | | | Citric | 140–160 | 150 |
| Propionic | | | ACIDS NOT ELUTED IN 172 FRACTIONS | | |
| Salicylic | | | p-Aminohippuric | | |
| p-Hydroxyphenylacetic | 8–14 | 10 | Ascorbic | | |
| Acetic | 10–20 | 14 | Aspartic | | |
| Acetoacetic | | | Creatine | | |
| Adipic | 16–24 | 19 | Glucuronic | | |
| Pyruvic | 19–27 | 23 | Glutamic | | |
| p-Aminosalicylic | 24–34 | 30 | Glyceric | | |
| Formic | | | Isocitric | | |
| Fumaric | | | Isocitric lactone | | |
| Phenylacetylglutamine | 31–35 | 32 | Kynurenic | | |
| Glutaric | 32–36 | 33 | Nicotinic | | |
| β-Hydroxy butyric | 33–44 | 36 | Oxalosuccinic | | |
| Lactic | 38–48 | 44 | Tartaric | | |
| Homogentisic | 40–55 | 48 | Quinic | | |
| Succinic | | | Uric | | |

[a] 3.7 ml. siphon.

Peak 1 is aromatic in character, the largest fraction apparently consisting of hippuric acid, according to the method of Fewster and Hall (16). The presence of acetic, formic, and lactic acids in peaks, 2, 3, and 5, respectively, were established by the method of Jones *et al.* (17) and by chromatography of their hydroxamates (18, 19).

Peak 4 is eluted in the same fraction range as phenylacetylglutamine which has been reported as a normal constituent of human urine (20, 21). More positive identification has not, as yet, been made.

Peak 6 is an unknown.

Peak 7 is a mixture containing oxalic and glycolic acids, as evi-

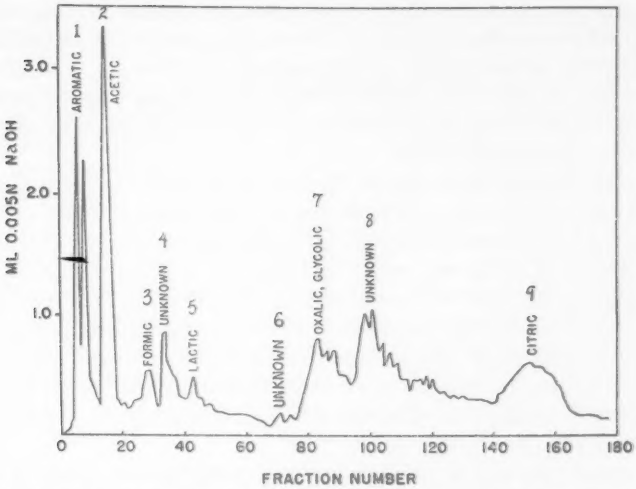


**Fig. 1.** A typical pattern obtained by the chromatography of the organic acid extract of 15 ml. of urine on a silica gel column. Milliliters of standard alkali required to titrate the acid in each fraction are plotted against the fraction number.

denced by their hydroxamates. The presence of *cis-* or *trans*-aconitic acid has not, as yet, been confirmed.

Peak 8 is an unknown not identical with malic acid.

Peak 9 is citric acid, confirmed by a method essentially that of Lugg and Overell (22).

Organic acid is titrated in the range immediately following lactic. A sharp peak, however, is not obtained. This fraction range corresponds to that of succinic and pyrrolidone carboxylic acids which are known to be present in urine. Organic acid is also titrated in the range of malic acid.

## DISCUSSION

The complexity of the aromatic acids present in urine has been shown by the work of Armstrong and co-workers (23), who have demonstrated the presence of 43 phenolic compounds by means of paper chromatography. It is presumed that most of these acids would be eluted in the first peak of the silica gel method. Osteux and Laturaze (24), by means of paper chromatography, have indicated the presence of 26 organic acids, while Nordmann and co-workers (25) have shown the presence of 19 organic acids in human urine. Loss of volatile

acids occurred as a result of a preliminary elution of the urinary acids from ion exchange resins. The elution ranges for the acids reported by the latter two groups are included in Table 2. The absence of a greater number of aromatic organic acids in the paper chromatograms of these groups is surprising in view of the large number described by Armstrong *et al.*

From the results of the paper chromatography of the acids titrated in each peak of Figure 1, it is apparent that there are two or more unknown acids present in significant quantities in urine. Nordmann *et al.* did not identify one acid which appeared persistently on their chromatograms, while Osteux and Laturaze found six unknowns. In several analyses an acid appeared between the known position of malic and citric acids which did not prove to be identical with either of these. An occasional run has demonstrated an acid in the immediate postcitric position not identified as citric, isocitric, or tartaric acids by paper chromatography.

The values for the elution ranges and peaks given in Table 1 are valid only for the 3.7 ml. siphon used in the procedure. Slight variation in the size of the siphon will cause a corresponding variation in these values. The *relative* position of elution, however, will remain unaltered. As Table 1 demonstrates, there may be considerable overlapping of individual acids which, together with the fact that biologically important acids are not eluted, constitute significant disadvantages in the use of silica gel partition chromatography on urine. The method, however, may be considered as a very useful analytical tool for separating the non-α-amino organic acids of urine into several groups which may be further studied by other means. It is possible to obtain a good separation of aromatic acids as a group, and to ob-

**Table 2.** Recovery of Organic Acids Carried Through Extraction Procedure Followed by Silica Gel Chromatography

| *Acid* | *μEq. present* | *μEq. found* | *% Recovery* |
|---|---|---|---|
| Lactic | 48.7 | 46.1 | 95 |
| Acetylsalicylic | 25.0 | 26.5 | 106 |
| Pyruvic | 44.5 | 21.8 | 49 |
| Oxalic | 89.5 | 91.5 | 102 |
| Malic | 67.1 | 70.0 | 104 |
| *Trans*-aconitic | 58.6 | 61.5 | 105 |
| α-Ketoglutaric | 171.0 | 131.0 | 77 |
| Fumaric | 24.1 | 22.2 | 92 |
| Hippuric | 10.1 | 9.8 | 97 |

serve quantitative changes in formic, acetic, lactic and citric acid concentrations.

## SUMMARY

1. A method for the extraction of non-α-amino organic acids from urine based on the work of Isherwood is described.

2. The relative position of elution of 42 biologically significant acids from silica gel columns is demonstrated. A number of important acids known to be present in urine are not eluted.

3. The partition chromatography of urinary organic acids reveals the presence of 9 peaks, of which 4 are known to be acetic, formic, lactic, and citric acids. A fifth peak contains glycolic and oxalic acids.

4. The aromatic acids are eluted principally in the first peak, which contains hippuric acids as a major constituent.

5. A minimum of two unknown organic acids are constantly present in relatively large concentrations in urine. Two additional acids appear frequently.

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# A Note on the Paper Chromatography of Hemoglobins

*F. Vella and J. W. H. Lugg*

IT WAS RECENTLY CLAIMED (1) that by paper chromatographic methods the hemoglobins prepared from a variety of human bloods—of normal adults, of the umbilical cord, and of subjects with sickle-cell anemia or Mediterranean anemia—could each be resolved into a number of molecular species. The methods employed involved the use, as solvents, of aqueous solutions of sucrose and salts, of pyridine and of *n*-butanol-acetic acid-water mixtures, alone in one-dimensional runs and in various combinations in two-dimensional runs, with filter paper as the sheet support.

In consequence of the persistent difficulties we had experienced in endeavoring to resolve hemoglobin mixtures by the methods of paper electrophoresis with incidental evaporation (2) and ion-exchange chromatography (3,4), we decided to explore the potentialities of the paper chromatographic methods, more especially those involving the use of aqueous sucrose and buffer solutions.

## METHOD

The hemoglobin solutions (which provided the initial spots) were prepared from heparinized blood samples, either by freezing and thawing to yield plasmolyzed whole blood, or by cytolyzing with water (with or without addition of toluene) the cells first separated by centrifugation and washing with isotonic saline, and then filtering or centrifuging the mixture.

The chromatograms were run at about 28°, the front advancing 12 cm. in about 40 min. when the solvent was a solution of sucrose, NaCl, or veronal buffer mixture. The condition of the blood pigment on

From the Department of Biochemistry of the University of Malaya, Singapore.

the sheet (Whatman No. 1 filter paper) was examined with the aid of a hand spectroscope immediately before and immediately after a run. The typical absorption bands of oxyhemoglobin, as such, on wet filter paper were readily discernible, but were largely lost if the paper were well dried out, and there was appreciable loss even in drying for 20 min. at 37° in a current of air.

## RESULTS

The results of our one-dimensional, ascending- front chromatography tests are summarized in Table 1. No differences were detected in the behavior of the hemoglobin solutions prepared from adult and umbilical-cord bloods, and there was no hint of any separation of hemoglobin species having been effected. Tests with descending-front technic and with veronal buffer at *p*H 8.6, citrate buffer at *p*H 6.5, and solutions of sucrose in either gave no promise of success. The results of two-dimensional tests followed closely enough the pattern that could be expected from the results of the one-dimensional tests, the chief complication arising from the previous mentioned effects of even partially drying the paper (this being necessary between the runs).

All in all, the findings have been disappointing. The damage suf-

Table 1. General Patterns of Behavior of Human Adult and Umbilical-cord Hemoglobins in One-dimensional, Ascending-front Chromatographic Tests

| *Solvent* | *Approx. composition* | *Movement of colored material* | *Color change* | *$HbO_2$ bands* |
|---|---|---|---|---|
| Aqueous sucrose solutions | 5-30 Gm./100 ml. | Streak $R_f$, 0.5-0.8 | Nil | Preserved |
| Aqueous NaCl solutions | 5-30 Gm./100 ml. | Streak $R_f$, 0.5-0.8 | Nil | Preserved |
| Veronal buffer *p*H 8.6 | 0.06 ionic strength | Streak $R_f$, 0.5-0.8 | Nil | Preserved |
| *n*-butanol-acetic acid-water | 20:3:7.5 by volume | Streak $R_f$, 0.0-1.0 | Brown | Lost |
| *n*-butanol-water | 20:10.5 by volume | Very slight $R_f$ ? | Nil | Preserved |
| Pyridine (moist) | – | Streak $R_f$, 0.0-1.0 | Brownish green | Lost |
| Lutidine-ethanol-water | 55:25:20 by volume | Streak $R_f$, 0.0-1.0 | Brownish green | Lost |
| Ethanol-water | 1:3 by volume | Streak $R_f$, 0.5-0.8 | Nil | Preserved |

fered by the hemoglobin in presence of the *n*-butanol-acetic acid-water mixture, the moist pyridine, and the lutidine-ethanol-water mixture was not entirely unexpected, but the remaining solvents, though leaving the hemoglobin apparently unaffected chemically, failed to yield satisfactory chromatograms.

## REFERENCES

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4. Huisman, T. J. H., and Prins, H. K., *J. Lab. Clin. Med.* **46,** 255 (1956).

the Clinical Chemist

## ROUTH ELECTED AACC PRESIDENT

JOSEPH I. ROUTH, Professor of Biochemistry at the School of Medicine State University of Iowa and director of the Clinical Biochemistry Laboratory of University Hospitals, was elected President of the American Association of Clinical Chemists to serve from July 1, 1957, to June 30, 1958. Professor Routh was born in Logansport, Indiana, and studied at Purdue and Michigan Universities, receiving his doctorate in biochemistry at the University of Michigan in 1937. He has been associated with Iowa State University since 1937 and in his present rank since 1951. His participation in activities for clinical chemistry has been varied and extensive, having served on the Executive Committee of the Association for several years and as vice-president for the 1956-57 term. He also organized the Mid-West Section and served on various committees of the Association. At present he is a member of the American Board of Clinical Chemistry and a member of the Board of Editors of CLINICAL CHEMISTRY. Until recently he was on the Committee on Clinical Chemistry of the American Chemical Society.

The other officers for the coming year are OLIVER H. GAEBLER, Edsel Ford Institute for Medical Research, Detroit, Vice-president; MAX M. FRIEDMAN, Lebanon Hospital, New York, National Secretary; and LOUIS B. DOTTI, New York, National Treasurer.

The members of the National Executive Committee for 1957-58 are ROBERT M. HILL, University of Colorado, Denver; FERRIN B. MORELAND, Baylor University, Houston; ELIZABETH POMERENE, St. Luke's Hospital, Cleveland; RICHARD J. HENRY, Bio-Science Laboratories, Los Angeles; and DAVID SELIGSON, Graduate Hospital of the University of Pennsylvania, Philadelphia. The ballots were tabulated and the election certified by the official tellers, Albert Hanok and Andre C. Kibrick.

## NOMINATING COMMITTEE

A Nominating Committee was elected by the membership, this committee being authorized to propose a slate of officers for the 1958-59 National Executive Committee.

The Nominating Committee consists of JOHN G. REINHOLD, University of Pennsylvania, Philadelphia, to serve as chairman; ROBERT L. DRYER, University Hospitals, Iowa City; GEORGE R. KINGSLEY, Veterans Administration Hospital, Los Angeles; GEORGE T. LEWIS, University of Mi-

ami, Coral Gables, Fla.; SAMUEL NATELSON, Rockford Memorial Hospital, Rockford, Ill.; MIRIAM REINER, District of Columbia General Hospital, Washington, D. C.; and HARRY SOBOTKA, Mount Sinai Hospital, New York. MAX M. FRIEDMAN, Lebanon Hospital, New York, was elected as alternate.

### NINTH ANNUAL MEETINGS

The Stated Annual Meeting of the American Association of Clinical Chemists will be held in conjunction with the meetings of the American Chemical Society in New York City during the week of September 9, 1957. At this writing the detailed program is not yet available. The sessions on clinical chemistry will be towards the latter part of the week, probably from Wednesday to Friday. The annual dinner has been scheduled for Thursday, September 12, at which time the sixth Ernst Bischoff Award will be presented. Other events to be scheduled include a symposium, some general sessions on clinical chemistry, and the annual membership meeting. Members are requested to consult *Chemical and Engineering News* for the complete program.

### SYMPOSIUM ON METHODOLOGY

The Cleveland Section of the American Association of Clinical Chemists, in cooperation with the Bunts Educational Institute, will sponsor a three-day session with the program title of "A Symposium of Clinical Chemistry Methods." The sessions will be held in Cleveland during November 13, 14, and 15. The topic for the first day will be "Liver Function Tests," for the second, "Electrophoresis," and the last day will be devoted to "Microchemistry and Enzyme Determinations." Guest speakers will include Drs. Miriam Reiner, District of Columbia General Hospital; Samuel Natelson, Rockford Memorial Hospital; John G. Reinhold, University of Pennsylvania; and Felix Wroblewski, Sloan-Kettering Institute, New York.

A detailed program will be published in the October issue of CLINICAL CHEMISTRY. In the meantime information and application blanks may be obtained by writing to Dr. Adrian Hainline, Jr., Cleveland Clinic, Cleveland 6, Ohio. Registration will be limited to 125 persons.

### REPORTS FROM THE SECTIONS

#### NEW YORK-METROPOLITAN

The following Resolutions were passed by the Executive Committee of the New York-Metropolitan Section:

*Resolution 1.* That a standing committee on ethics and standards be appointed.

*Resolution 2.* The functions of such a committee to be the offering of its services in problems of clinical chemistry to the Board of Health and the various medical societies of the New York City area, as well as any laboratories who desire this consultation service.

A. SAIFER
*Secretary-Treasurer*

### CLEVELAND

The Cleveland Section met on June 4, 1957, at which time Dr. Lena Lewis of the Cleveland Clinic Foundation spoke on electrophoresis. The meetings for the past year were well attended and an active program is anticipated for the coming year.

Officers elected for the 1957-58 term are ADRIAN HAINLINE, JR., Cleveland Clinic, chairman; IRVING SUNSHINE, Western Reserve University, vice-chairman; and STANLEY LEVEY, University Hospitals of Cleveland, secretary-treasurer.

### TEXAS

The spring meeting of the Texas Section was held on June 15, 1957, at the Methodist Hospital in Houston. During the morning session papers were read by John K. Kirby of the Clinical Pathological Laboratory of Austin on "Plasma Iron Concentration in Bank Blood," and by Paige K. Besch of the Southwest Foundation for Research and Education of San Antonio on "A Spectrophotometric EDTA Titration Method for Determining Calcium." Other methods for calcium and iron and iron binding capacity were discussed.

After luncheon in the hospital cafeteria, the afternoon session consisted of additional discussions of methods for chloride, transaminases, etc. The business meeting at 2:30 P.M. was followed by social sessions and visits to points of interest in Houston.

### PHILADELPHIA

Statistical analysis of chemical determinations on pooled serum was the topic discussed at the forty-fourth scientific meeting of the Philadelphia Section of the American Association of Clinical Chemists. The meeting was held on March 26, 1957, at the Hospital of the University of Pennsylvania.

A review of statistical methodology was given by Dr. Samuel I. Askovitz of the University of Pennsylvania School of Medicine and the Albert Einstein Medical Center. Discussed were measures of central tendency, measures of dispersion, and criteria for rejection of doubtful observations. Of particular interest were measures of dispersion more easily calculated than the well known standard deviation. Discussion centered around the need for better standardization of some technics, and the question of how much less should be the analytical dispersion for a given substance than the physiological dispersion in order for laboratory results to be clinically useful.

The forty-fifth scientific meeting of the Philadelphia Section was held on April 23, 1957, at the Hospital of the University of Pennsylvania. In a talk by Dr. Bernard Shapiro of the Albert Einstein Medical Center, uric acid methods and results were compared with a new technic. The method introduced by the speaker consists of the isolation of uric acid on Dowex 2XR resin, removal of interfering substances, and elution with weak hydrochloric acid. The eluted material was measured by ultraviolet absorption, enzymatically, and colorimetrically by the Folin-Brown method. Recoveries were noted between 89 and

107 per cent, and comparisons were made among the methods.

At the business meeting that followed new officers were elected as follows: Margaret Vanderau, Presbyterian Hospital, Philadelphia, chairman; Howard W. Robinson, Temple University, vice-chairman; Peace Paubionsky, Abington Memorial Hospital, secretary-treasurer.

PEACE PAUBIONSKY

### MEETINGS OF THE ASSOCIATION OF CLINICAL BIOCHEMISTS

The General Meeting of the Association of Clinical Biochemists of the United Kingdom was held jointly with the Biochemical Society at the Royal Victoria Infirmary, Newcastle-upon-Tyne, England, on Saturday, June 29, 1957. The scientific program follows:

#### SYMPOSIUM ON MUCOPROTEINS

A. J. ANDERSON and N. F. MACLAGAN (London): Urinary colloids and precipitation reactions.

J. W. KEYSER (Cardiff): Factors influencing the serum level of mucoproteins.

BESS M. STILL (London): Mucoprotein as an activator of the plasminogen-plasmin enzyme system.

A. L. LATNER (Newcastle-upon-Tyne): Intrinsic factor mucoprotein.

#### COMMUNICATIONS

A. J. SMITH and L. B. STRANG (Newcastle-upon-Tyne): The presence of α-hydroxybutyric acid in the urine of a patient with phenylketonuria.

R. F. FLETCHER, A. C. FRAZER, H. G. SAMMONS, and B. SHAW (Little Bromwich): Further observations in the study of steatorrhoea.

H. A. DUDLEY, J. S. ROBSON, MAUREEN SMITH, and C. P. STEWART (Edinburgh): Metabolic changes following adrenalectomy.

M. R. CHOUDHURY, G. A. SMART, and R. B. THOMPSON (Newcastle-upon-Tyne): Measurement of iron absorption by the use of $Fe^{59}$.

W. FORSHALL, BETTY IGGO, and C. P. STEWART (Edinburgh): Observations on the metabolism of ascorbic acid in rheumatoid arthritis.

A. L. LATNER and A. J. SMITH (Newcastle-upon-Tyne): The transaminase alkaline phosphatase ratio in the differential diagnosis of jaundice.

P. TRINDER (Sunderland): A rapid method for the estimation of calcium in small quantities of serum.

#### DEMONSTRATIONS

F. T. FARMER (Newcastle-upon-Tyne): A high sensitivity scintillation counter for *in vitro* measurements.

L. MOLYNEAUX and E. A. PASK (Newcastle-upon-Tyne): Contin-

uous control of endexpiratory $pCO_2$ and pulmonary ventilation.

S. G. Owen and G. A. Smart (Newcastle-upon-Tyne): Tannic acid haemagglutination method in the demonstration of antibodies.

A. J. Smith (Newcastle-upon-Tyne): The demonstration of urinary phenylpyruvic acid by paper chromatography.

W. Still and E. H. Boult (Newcastle-upon-Tyne): Electron microscopy of fibrin.

A. H. Zaki (Newcastle-upon-Tyne): Starch gel electrophoresis of serum.

# BOOK REVIEWS

**Autoradiography in Biology and Medicine.** G. A. Boyd. (*New York, Academic Press,* 1955, 399 pp., $8.80)

This book is written for the investigator who has never used the autoradiographic technic. Here the reader will find a wide range of information as to the material needed in order for him to gain the most information from proposed experiments.

The book is divided into three sections—Theory, Technics, and a rather extensive Bibliography. Though Dr. Boyd seems to devote much space to the use of this technic after histologic preparation of plant and animal tissue, the first 163 pages, devoted to theory, which encompasses both photographic and radiation theory, is applicable to all users of autoradiography and will be of great value.

Part II, which the author comments on as being written in "cook book style," was found to be well done. The procedures are given stepwise, with a well-written Discussion covering certain refinements and observations either from the author's experience or noted in the literature. The methods covered deal chiefly with histologic preparations and a procedure for airborne and other particulate matter, such as sand silt and dust. Yet there are other applications which would come under the scope of biology and medicine which the author does not include, such as application to chromatograms of biologic fluids, even though the initial preparations may be under the scope of biochemistry.

*Metropolitan Hospital, New York* H. D. Appleton

# ABSTRACTS

*Editor:* Ellenmae Viergiver. *Contributors:* Joseph S. Annino, Gladys J. Downey, Clyde A. Dubbs, Alex Kaplan, Margaret M. Kaser, Miriam Reiner, Herbert Thompson

**Improved method for determination of calcium and magnesium in biologic fluids by EDTA titration.** M. H. Carr and H. A. Frank (*Veterans Administration Hospital, Kansas City, Mo.*).

A procedure is described for the analysis of calcium and magnesium in serum, spinal fluid, urine, gastric secretion, and bile. This method is a modification of previously published procedures and is based upon the separation of calcium from magnesium by quantitative precipitation of calcium oxalate followed by titration of each of the ions separately with ethylenediamine tetraacetate (EDTA) using eriochrome black T as the indicator. The results compare favorably with those obtained by conventional procedures, and the method has the advantage of being simpler to perform. The authors feel it is suitable for use in the clinical laboratory.—*Am. J. Clin. Pathol.* **26,** 1157 (1956).

(H. E. T.)

**Turbidimetric determination of proteins in dilute solution.** P. Morand and J. Dufau-Casanabe.

The turbidity produced by adding nitric or sulfosalicylic acid to cerebrospinal fluid depends not only on total protein concentration but also on the nature and relative amounts of the various component proteins. The practice of pooling serum from individuals to standardize the turbidimetric procedure can result in large errors since the serum pool is not necessarily representative of an individual serum or spinal fluid.—*Bull. soc. pharm. Marseilles* **4,** 53 (1955).

(E. V.)

**Effect of high salt concentrations on color production of the biuret reaction for protein analysis.** H. L. Rosenthal and T. Kawakami (*Rochester General Hospital, Rochester, N. Y.*).

The violet color produced by interaction of copper ions in alkaline solution (biuret) is altered by concentrated solutions of inorganic salts. When comparable amounts of salts are included in the standard solutions of protein, the results obtained with the biuret method closely parallel those obtained with the classic Kjeldahl procedure.—*Am. J. Clin. Pathol.* **26,** 1169 (1956).

(H. E. T.)

**Simplification of the spectrophotometric determination of glutamic-oxalacetic transaminase in blood serum.** C. Sobel, S. Berkman, and N. Swabb (*Bio-Science Laboratories, Los Angeles, Calif.*)

By substituting a 0.0005N potassium dichromate solution for the serum blank, as employed in the method of Karmen, costly reagents are saved and 3 determinations may be made simultaneously in the Beckman Model B Spectrophotometer.—*Am. J. Clin. Pathol.* **26,** 1477 (1956). (H. E. T.)

**A colorimetric method for transaminase in serum or plasma.** W. W. Umbreit, G. R. Kingsley, R. R. Schaffert, and H. Siplet. (*Merck Institute for Therapeutic Research, Rahway, N. J., Veterans Administration Center, Los Angeles, Calif., and Fels Research Institute, Philadelphia, Pa.*)

Transaminase catalyzes the reaction between aspartate and α-ketoglutarate to form oxalacetate and glutamate. The oxalacetate is decomposed chemically to pyruvate which then reacts with dinitrophenylhydrazine to form a hydrazone. By treatment with alcoholic KOH the hydrazone is converted to a colored compound which is read at a wave length 450 and 520 mμ. Small amounts of transaminase are readily detected and can be quantitatively recovered.—*J. Lab. Clin. Med.* **49,** 454 (1957). (G. J. D.)

**Effect of aspirin administration on serum glutamic oxaloacetic and glutamic pyruvic transaminases in children.** C. Manso, A. Taranta, and I. Nydick (*Cornell University Medical College, New York, N. Y.*)

The concentration of SGO-T and SGP-T were determined following the administration of aspirin or sodium salicylate to 23 children convalescing from rheumatic fever. Fourteen patients received aspirin or sodium salicylate in daily doses of 0.6 to 1 Gm./15 lb. body weight for at least 4 weeks. SGO-T was determined during a control period and then twice weekly. In the other 9 patients on a similar salicylate regimen, both SGO-T and SGP-T were measured. All patients had normal enzyme activities during the control period. During salicylate therapy 8 of the first group showed elevations of SGO-T above 50 units, the upper limit of normal. The highest was 146 units. In the second group, 6 children showed similar elevations of both SGO-T and SGP-T. In one child, who had had infectious hepatitis 18 months earlier, the SGO-T reached 540 units and the SGP-T 900 units. Increased enzyme activity usually occurred only after several weeks of salicylate administration. There were no consistent relationships between serum enzyme activities and serum salicylate levels. It is believed that the liver was the source of the increased enzyme activity.—*Proc. Soc. Exptl. Biol. Med.* **93,** 84 (1956). (M. M. K.)

**A titration method for the determination of calcium in serum using a new indicator.** M. A. Andersch (*University of Maryland Medical School. Baltimore, Md.*)

A new indicator, calcein, is proposed for the titration of calcium in serum with ethylenediaminetetraacetic acid. This indicator is specific for calcium in the presence of magnesium in a highly alkaline *p*H, and the color change from yellow green in the presence of calcium ion to brown in its absence is said to be easily detected. Values obtained by this method were comparable to those obtained by the Clark-Collip procedure.—*J. Lab. Clin. Med.* **49,** 486 (1957). (G. J. D.)

**A new colorimetric reagent for microdetermination of ammonia.** W. E. Stone (*University of Wisconsin Medical School, Madison, Wisc.*)

A method is described for the determination of 0.05 to 0.5 $\mu$m of ammonia, which is more rapid and convenient than the usual microtitration and has at least equal precision. Ammonia was found to be oxidized stoichiometrically by hypobromite in concentrations at least as low as 0.03 $\mu$m. A measured excess of hypobromite is added and the excess is determined by its ability to decolorize phenosafranin.

Microdiffusion is carried out in a Conway unit and the ammonia is absorbed in 0.01N $H_2SO_4$ in the central chamber. The contents of this chamber are quantitatively transferred to a colorimeter tube with water. A solution of bromine in borate buffer is added, followed by phenosafranin 2 minutes later. The resulting solution is diluted and the optical density is determined at 515 m$\mu$. The procedure is calibrated with standard ammonium sulfate solutions and appropriate corrections or blanks are made in each series of determinations.—*Proc. Soc. Exptl. Biol. Med.* **93,** 589 (1956). (M. M. K.)

**Effects of azetazoleamide (Diamox) upon blood sugar of normal and diabetic patients.** A. J. Grossman and R. C. Batterman (*New York Medical College, Bird S. Coler Memorial Hospital and Home for the Aged, New York, N. Y.*).

Subjects for this study consisted of 20 normal and 20 diabetic individuals. Fasting levels and the blood sugar 3 hours after the administration of 500 mg. of Diamox orally were determined by the Folin-Wu method. Control determinations without the drug were done a week before or after this procedure. The drug was without influence on the blood sugar level. Three normal and 3 diabetic patients were given 500 mg. of Diamox daily for 3 weeks. There was no difference in fasting blood sugar values determined at weekly intervals between treated and untreated subjects. Standard oral glucose tolerance tests with and without Diamox were performed on 3 normal and 3 diabetic subjects, and again no significant differences attributable to the drug could be demonstrated.—*Proc. Soc. Exptl. Biol. Med.* **93,** 255 (1956). (M. M. K.)

**A micromethod for the determination of salicylic acid in plasma.** S. Chiang and S. Freeman (*Northwestern University Medical School, Chicago, Ill.*).

A micromethod for the determination of salicylic acid has been developed which consists of a direct separation of salicylic acid from plasma by simple ascending paper chromatography with ethanol as the resolving solvent, followed by the colorimetric estimation of the salicylic acid by extraction with water and ferric nitrate reagent. Optical density is read in a Beckman spectrophotometer at 540 mμ in micro cells. The determination can be done on as little as 0.02 ml. of plasma and is sensitive to 0.5 μg. of salicylic acid.—*J. Lab. Clin. Med.* **49,** 481 (1957). (G. J. D.)

**Manual of American Society of Clinical Pathologists workshop on glucose (condensed version).** F. W. Sunderman, B. E. Copeland, R. P. MacFate, V. E. Martens, H. N. Naumann, and G. F. Stevenson (*U. S. Naval Medical School, Bethesda, Md.*).

The Sunderman-Fuller macroprocedure and the Sunderman microprocedure for blood glucose are described. Procedures for the identification of urine sugars include yeast fermentation, paper chromatography, phenyl hydrazone formation, and polarimetry. Special consideration is given to the correct diagnosis of essential pentosuria.— *Am. J. Clin. Pathol.* **26,** 1355 (1956). (H. E. T.)

**Blood corticoids: their measurement and significance.** J. J. Gold (*Michael Reese Hospital, Chicago, Ill.*).

A review of the status of blood corticoid determination in the appraisal of adrenal function is presented. Background studies, methodology, and clinical applications are discussed.—*J. Clin. Endocrinol. and Metabolism* **17,** 296 (1957). (M. R.)

**Demonstration of a marked Bence Jones proteinemia.** J. I. Morton and H. F. Deutsch (*University of Wisconsin, Madison*).

Serum from a patient with multiple myeloma was studied by electrophoretic and ultracentrifugal technics. The data are believed to offer conclusive evidence of the presence of Bence Jones protein with a molecular weight of 35,000 to 40,000.—*Proc. Soc. Exptl. Biol. Med.* **93,** 402 (1956). (M. M. K.)

**New dye method for direct photometric determination of calcium.** G. R. Kingsley and O. Robnett (*Veterans Administration Center, Los Angeles, Calif.*).

The authors report an extremely simple and accurate method for the direct photometric determination of calcium in 0.1 ml. of serum and in urine and

spinal fluid samples. The purple dye, disodium-1-hydroxy-4-chloro-2,2-diazobenzene-1,8-hydroxynaphthalene-3,6-disulfonic acid, becomes red in the presence of calcium, and if the reaction is carried out in a strongly alkaline solution, there is no interference from magnesium. The results agree well with established calcium methods.—*Am. J. Clin. Pathol.* **27,** 223 (1957). (H. E. T).

**A colorimetric procedure for the determination of blood ammonia.** D. G. Nathan and F. L. Rodkey (*Harvard Medical School, Boston, Mass.*)

A microdiffusion technic for the measurement of blood ammonia in a trichloroacetic acid filtrate is carried out in a modified Seligson unit by the photometric ninhydrin method. The method is more sensitive than nesslerization and more convenient than microtitration. The procedure is reproducible, and recovery of added ammonia is quantitative.—*J. Lab. Clin. Med.* **49,** 779 (1957).
(G. J. D.)

**The determination of phosphorus and phosphatase with N-phenyl-p-phenylenediamine.** R. L. Dryer, A. R. Tammes, and J. I. Routh (*State University of Iowa, College of Medicine, Iowa City, Iowa*).

The method presented here is a modification of the classical technic for the determination of phosphorus. A solution of *N*-phenyl-*p*-phenylenediamine hydrochloride (*p*-semidine hydrochloride) is used to reduce the phosphomolybdic acid to produce a stable color. Technics are presented for determination of phosphate in tissue and serum and of alkaline phosphatase. The advantages of semidine over other reducing agents are the increased sensitivity of the color reaction and the stability of the color formed.—*J. Biol. Chem.* **225,** 177 (1957). (J. S. A.)

**Colorimetric measurement of serum glutamic pyruvic transaminase.** F. Wroblewski and P. Cabaud (*Sloan-Kettering Institute, New York, N. Y.*)

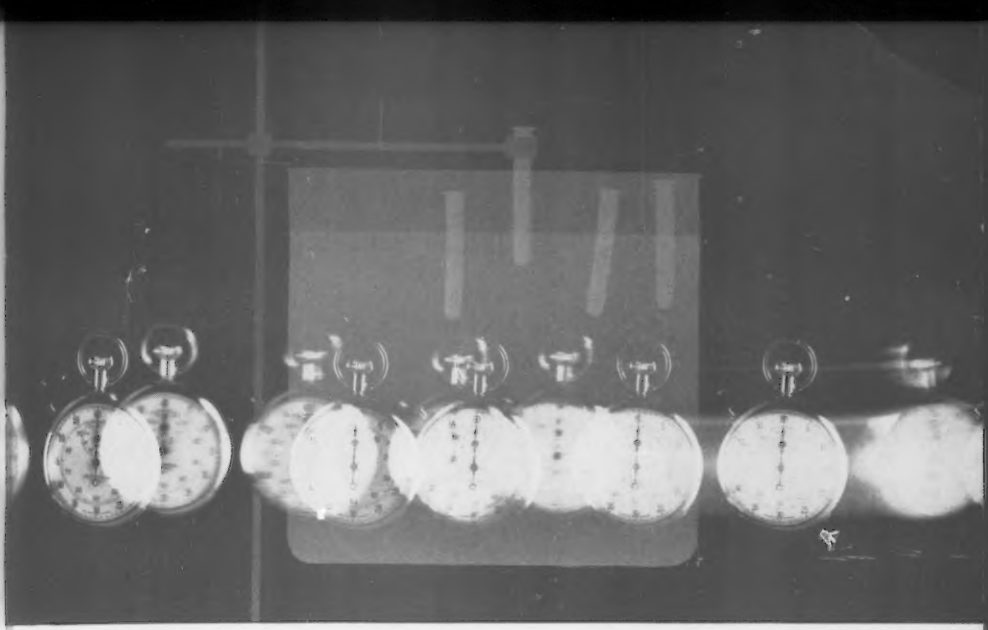
Details of a colorimetric procedure for assaying serum levels of glutamic pyruvic transaminase are presented. The simultaneous measurement of serum glutamic oxalacetic transaminase and glutamic pyruvic transaminase appears to provide a means of distinguishing acute hepatocellular injury from that which is chronic.—*Am. J. Clin. Pathol.* **27,** 235 (1957). (H. E. T.)

**Determination of catecholamines (adrenalin and noradrenalin) in urine and tissue.** C. Sobel and R. J. Henry (*Bio-Science Laboratories, Los Angeles, Calif.*).

Fluorimetric methods are presented for the determination of adrenalin and noradrenalin, or their sum, in urine and in tissue. The method is relatively simple and rapid.—*Am. J. Clin. Pathol.* **27,** 240 (1957). (H. E. T.)

# Clinical Chemistry Session at Annual Meetings of American Association for Advancement of Science

September 15, 1957, is the deadline for receipt of abstracts of papers to be presented at the session of the American Association of Clinical Chemists, during the AAAS Meeting to be held in Indianapolis, Ind., December 26-31, 1957. Abstracts should be sent to ALBERT E. SOBEL, Department of Biochemistry, Jewish Hospital of Brooklyn, Brooklyn 38, N. Y.



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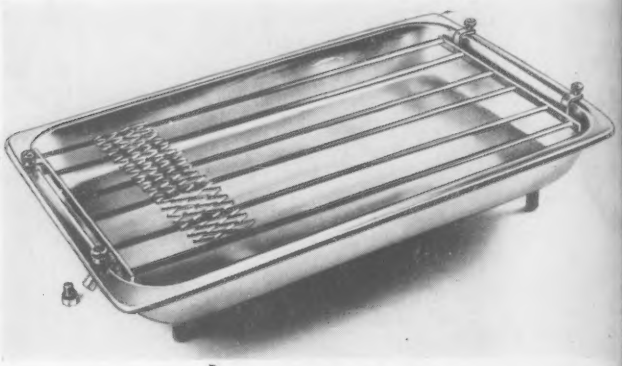

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